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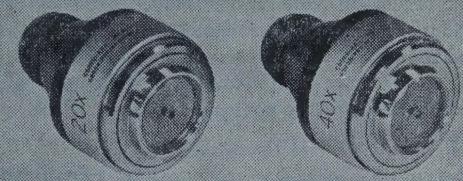
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THE PERMEABILITY OF THE SHELL OF THE EGG
OF *ACHETA COMMODUS* WALKER*
(ORTHOPTERA, GRYLLIDAE)

BY T. O. BROWNING

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(Received 11 July 1959)

INTRODUCTION

During development the eggs of *Acheta commodus* almost double their original weight by absorbing water. Virtually all this increase occurs during the third and fourth day of incubation at 27° C. (Browning, 1953). In all insect eggs that have been studied and found to absorb water, the period during which water is taken in is always restricted to a specific, brief stage of embryogenesis (Edney, 1957).

The eggs of *Phyllopertha horticola* absorb water only between the third and eighth days at 20° C. and Laughlin (1953, 1957) explains this by assuming that the membranes, having been impermeable to water at first, become permeable on the third day and water enters the eggs in response to an osmotic gradient. On the eighth day the membranes again become impermeable, or alternatively water ceases to flow in because of the increased hydrostatic pressure in the egg. Laughlin supported this theory with measurements of the rate at which eggs lost water in dry air at different stages of development and by observations on the morphology of the membranes of the egg. But he added that his physical explanation would not account for the whole process. Slifer (1958 and earlier papers) also considers the entry of water into the eggs of *Melanoplus differentialis* to be controlled by impermeable layers in the shell which later become permeable.

Most authors accept an alternative theory that the movement of water into and out of eggs is under the control of specialized cells (e.g. the hydropyle cells of Acrididae (Slifer, 1938)). The evidence on which this theory rests is that: (i) the eggs of *Locustana* will not absorb water if left in an atmosphere of nitrogen (Mattée, 1951); (ii) the rate of water uptake in some eggs is markedly influenced by temperature (Banks, 1949; Browning, 1953); (iii) the course of water absorption seems to be closely related to the stage of development of the embryo (cf. Edney, 1957); and (iv) dead or injured eggs either do not swell or in some cases may swell until they burst. But in our opinion none of this evidence necessarily conflicts with Laughlin's theory.

* Previously called *Gryllulus commodus* (Browning, 1952, 1953).

We have tested Laughlin's theory using deuterium oxide, and assuming that an egg that is permeable to deuterium oxide may reasonably be considered to be permeable to water also.

METHODS

Freshly laid eggs of *Acheta* were kept at 12.5° C. for about a month to permit diapause development to be completed (Browning, 1952). During this period the eggs do not change appreciably in weight (Browning, 1953). The eggs were then carefully dried by rolling them on paper tissue and leaving them exposed to the air for about 20 min. They were then put on moist filter-paper in a sealed Conway vessel and incubated at 27° C. When water from a sample of eggs was to be collected the eggs were removed from the Conway vessel, dried as before, washed quickly in three changes of distilled water, dried again, placed in the chamber of the apparatus and crushed with a blunt seeker.

The apparatus used for collecting the water consisted of a spherical chamber connected via a ground glass joint and a high-vacuum stopcock to a long, narrow U-tube cold trap. This led, via another high-vacuum stopcock to a 'Speedivac' pump capable of producing a vacuum of 0.1μ Hg. A cold trap and a McLeod pressure gauge were also placed in the vacuum line. When a sample of water was to be collected the cold trap in the vacuum line was frozen with liquid air and the system evacuated to the stopcock separating the sample chamber from the collecting U-tube. The sample chamber was sealed on to the apparatus and frozen. The stopcock was then opened, the whole system was evacuated, and the stopcock was turned off again. The collecting U-tube was then frozen, the stopcock was opened and the sample chamber was allowed to thaw at room temperature. Water evaporating from the sample chamber was collected for about an hour. About 1 mg. of water was condensed in the U-tube. Both stopcocks were then closed, the apparatus removed from the vacuum line and within 24 hr. the water was analysed in a mass-spectrometer.

The mass-spectrometer used was one designed for handling samples of gas for biological assays (Cooke-Yarborough & Russell, 1953) with a designed accuracy of $\pm 2\%$ in determinations of isotope ratios. With samples of water the accuracy was poor for two reasons. First, water reacts with the tungsten filament of the ion source, and secondly, the instrument has a high water background. In this type of design it is not possible to remove water adsorbed on the surface of the vacuum chamber, so that exchange between the sample and this adsorbed layer may give rise to very large 'memory' effects.

The procedure in handling the sample was designed to minimize this; the sample was admitted to the ion source of the spectrometer for some minutes before taking a reading, so that the water adsorbed in the chamber approached the composition of the sample and constant readings could be obtained. The sample tube was attached to the gas inlet of the mass-spectrometer and the gas handling lines were evacuated, the sample meanwhile being frozen with liquid air. When the sample tube was opened to the gas reservoir of the instrument any rise in pressure in the reservoir was

attributed to air; if this was considerable the sample was discarded, otherwise it was allowed to vaporize into the reservoir, from where it was admitted to the instrument.

We measured the ratio of the peak heights at masses 18, 19 and 20 atomic mass units. The instrument was calibrated with solutions of known dilution from an ampoule of 99% deuterium oxide. The calibration curve (Fig. 1) shows the large scatter of the results. However, the precision was high enough for the purpose of our argument.

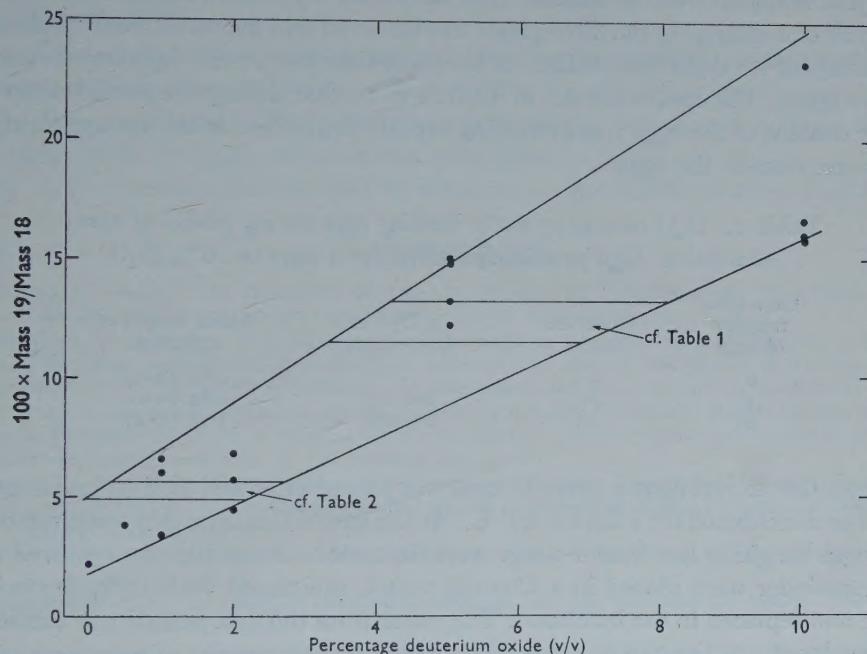


Fig. 1. Calibration diagram used for estimating deuterium-oxide concentrations in this study.
All values in Tables 1 and 2 lie in the areas indicated in the figure.

RESULTS

A large number of eggs was taken and placed in a Conway vessel on filter-paper well moistened with a 10% solution of deuterium oxide. Samples of eggs were removed each day for 4 days and the water was extracted and analysed. From Table 1 it can be seen that the deuterium-oxide content of the eggs increased during

Table 1. Changes in concentration of D₂O in eggs incubated in 10% solution of D₂O

Days at 27° C.	Observed ratio*	% D ₂ O in eggs†	Mean weight of eggs:mg.
0	—	—	0.60 ± 0.04
1	12.7	3.8–8.0	0.61 ± 0.04
2	11.5	3.3–7.2	0.64 ± 0.03
3	13.2	4.0–8.3	0.81 ± 0.21
4	12.5	3.7–7.8	1.14 ± 0.23

* Ratio of 100 × mass 19/mass 18.

† Read from Fig. 1.

the first 24 hr. when their weight was remaining constant, and that after this there was little change in the concentration of deuterium oxide in the eggs. This experiment was repeated with essentially similar results, except that the mass ratio of the water extracted from the eggs fluctuated around 10 (cf. Fig. 1) over a period of 7 days.

On the second day of the first experiment twenty-five eggs were removed, washed and placed in another Conway vessel on filter-paper which was moistened with just sufficient water to soak it. They were then replaced in the incubator and at intervals one-quarter of the filter-paper was removed and the water from it collected and analysed for deuterium oxide. At the same time four or five eggs were removed and weighed. The results set out in Table 2 show that during the period when the water content of the eggs was increasing rapidly deuterium oxide was appearing in the water outside the eggs.

Table 2. D_2O content of water bathing eggs during period of water absorption. Eggs previously bathed for 2 days in 10% D_2O

Days after transfer of eggs	Observed ratio	% D_2O in filter-paper	Mean weight of eggs:mg.
0	—	—	0.64 ± 0.03
1	4.9	0.6-2.3	0.81 ± 0.12
2	5.6	0.9-2.8	1.10 ± 0.27

In another experiment a group of eggs was placed in a Petri dish on moist filter-paper and incubated for 4 days at 27° C. At the end of this time they were removed and eggs weighing less than 1.0 mg. were discarded. A sample was removed and the remainder were placed in a Conway vessel, moistened with 10% deuterium oxide and replaced in the incubator. The water from the first sample was extracted and analysed. At the end of 1 day and 4 days, further samples of eggs were taken and the water from them collected and analysed. The results of this experiment showed that after 1 day the mass ratio of the water from the eggs had risen from 2.2 to 6.5 and had reached 10.3 by the end of 4 days. During this time there was no significant change in the weight of the eggs.

DISCUSSION

The objection may be raised that no net transfer of water had taken place when the eggs were not gaining weight, and that the deuterium found inside the eggs had got there by exchange with hydrogen atoms in the membrane. This cannot be disproved but seems unlikely.

Linderstrøm-Lang (1955) has shown that the hydrogen atoms in proteins in solution may exchange with deuterium from deuterium oxide very rapidly, but that the exchange is limited to labile hydrogen atoms. The membrane of the egg is a tough, modified protein, insoluble, chemically inert and of very high molecular weight. Such a protein has very few reactive groups, and the number of labile hydrogen atoms which could exchange with deuterium would be so low that

exchange could hardly account for the observed rate of movement of deuterium oxide across the membrane. The membrane is certainly permeable to O₂ and CO₂ and the simple explanation of our results is that it is permeable to water also. Apparently water may move across the membranes of the egg in both directions whether the water content of the egg is increasing or not.

SUMMARY

1. The passage of deuterium oxide across the egg membranes of *Acheta commodus* has been studied.
2. Deuterium oxide enters the egg at times when its weight is constant and leaves the egg at times when its weight is increasing as a result of uptake of water.
3. It is considered unlikely that these findings can be accounted for by simple exchange with hydrogen atoms in the membrane.
4. It is concluded that the shell membranes are permeable to water at all times.

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INDUCTION OF DIAPAUSE IN *ERIOISCHIA BRASSICAE* BOUCHÉ (DIPT., ANTHOMYIIDAE)

By R. D. HUGHES

National Vegetable Research Station, Wellesbourne, Warwick*

(Received 31 August 1959)

INTRODUCTION

A culture of cabbage rootfly (*Erioischia brassicae*) has been maintained for 4 years in a heated glasshouse at Wellesbourne. During this period a series of generations could be reared between late March and September, the pupal stage of each generation occupying 10–20 days. In the autumn and winter, however, breeding was interrupted because the pupal stage of generations reared during this period was prolonged to between 60 and 200 days.

Under field conditions in southern England the rootfly passes through three generations in a year; in the first two the pupal stage normally occupies 2 weeks, whilst in the third (autumn) generation the pupae remain dormant over the winter. The stage then lasts about 200 days and gives rise to flies in the spring of the following year.

The occurrence of a dormant period, similar to the natural overwintering condition, in pupae raised and kept in a heated glasshouse suggests that these pupae were in a state of diapause induced by variations of the environment other than temperature. The work of Lees (1955) and others indicated that daylength during development was a factor which might influence the time spent in the pupal stage.

METHODS OF CULTURE

The procedure used for culturing cabbage rootfly was briefly as follows. The eggs were collected from a cage containing the adult flies and were placed in their natural oviposition site on the soil around the base of the experimental host plants. Young actively growing turnips were used as hosts, successive batches of plants being raised in pots throughout the year. After 3 or 4 days the eggs hatched and the young larvae started to feed on the root cortex.

The turnips were grown in a heated glasshouse in which there was a daily cycle of temperature between approximately 10° and 20° C. throughout the year, although in summer the upper limit was occasionally exceeded. The larvae fed on the turnips for about 4 weeks, and when fully developed left the root to pupate in the soil. Within a week of their formation the pupae were washed out of the soil and put into gauze-topped glass jars half filled with damp vermiculite. The jars were stored at 20° C. and the flies that emerged into the upper part of the jars were removed each day.

* Now at the Entomology Division, C.S.I.R.O., Canberra, Australia.

OBSERVATIONS ON THE CULTURE RECORDS

The emergence of the flies was recorded from more than forty experimental generations reared in this way. Fig. 1 shows the emergence curves of seven representative populations. They may be divided into two primary groupings according to the length of the emergence period. Group A (two curves) have a short pupal period and a high percentage emergence, whilst group B (three curves) have a long pupal period and a low percentage emergence. The two remaining curves were intermediate in both respects.

The low percentage emergence within the group B populations was a result of the prolonged and continuous storage of the pupae at a temperature of 20° C. If such

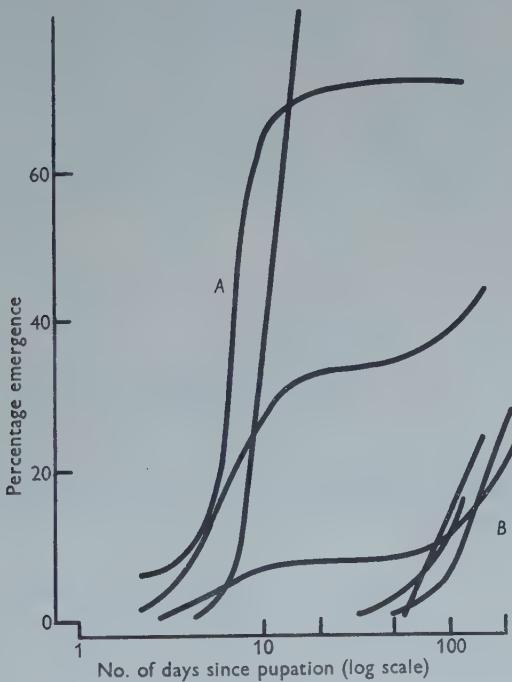


Fig. 1. Emergence curves of culture populations of pupae showing short and long and intermediate pupal stages.

pupae were given 6 weeks' cold treatment at 4° C. during storage, then about 60% of the population emerged.

Group A curves are typical of populations reared in the spring and summer, and group B of those raised in the autumn and winter. The intermediate type of curve occurred with populations raised in early spring and early autumn. When the proportion of individuals in each generation which enter into a prolonged pupal stage is plotted against the time of year during which development occurred, the continuous nature of the annual changes of this proportion can be seen. This effect

is shown graphically in Fig. 2, each population being represented by a thick horizontal line starting at the time when the eggs were placed on the turnips and ending with the time when the pupae were washed out from the pot soil. The upper abscissa of Fig. 2 gives the annual cycle of daylength to show that the change over from long to short pupal periods occurs in generations which have their larval development when the daylengths are the same. From this culture data it can be calculated that half the individuals of a generation would have gone into a prolonged pupal stage if the larval stage developed during a period when the mean time between sunrise and sunset was just over 14 hr.

These observations suggest that the prolonged pupal stage may be a facultative diapause induced by the daylength at some stage of the larval development.

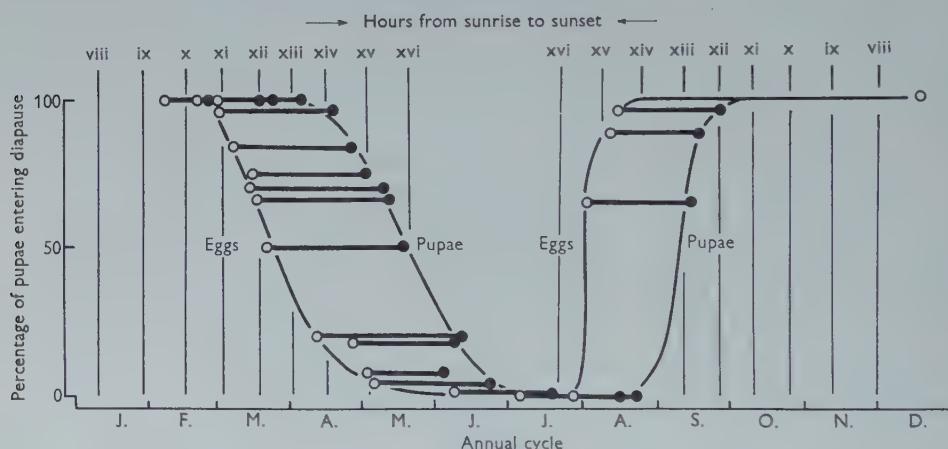


Fig. 2. Proportion of individuals entering a prolonged pupal stage compared with the time of year when development of the larvae took place. Key: ○, times when eggs were placed on plants; ●, times when pupae were collected.

DEMONSTRATION OF DAYLENGTH AS A STIMULUS TO DIAPAUSE

To investigate further the action of daylength during the larval stage on the occurrence of pupal diapause, and to determine whether the perceptive period is confined to a shorter time than the whole developmental period, the following experiment was carried out. Two daylength treatments were used: (a) normal daylength, which increased from 12 to 13·5 hr. during the experiment, as the short day, and (b) this normal daylength supplemented throughout the night by a 500 W. mercury vapour lamp giving 250 f.c. at the plant, as the long day.

A group of thirty eggs was placed around each of twelve turnip plants which were at a similar stage of growth. The schedule of the combinations of short-day and long-day treatments given to each plant is shown in Table 1. From the table it will be seen that the percentage of pupae which entered diapause varied greatly with the treatments. The proportion of diapausing pupae was progressively reduced as the number of long days increased and long days at the beginning of larval development were much more effective than long days towards the end of the larval stage (compare

plants 5 and 6 with plants 8 and 9). Long days of 24 hr. light tend to prevent diapause as long as a certain proportion occur during the first 4 weeks of larval life. As few as 7 long days appear to be sufficient to prevent the majority of the pupae from going into diapause.

Table 1. *The effect of different combinations of short- and long-day treatments on the proportion of *Erioischia brassicae* individuals entering a prolonged pupal stage*

Plant	Schedule	Proportion of diapausing pupae (%)	No. of pupae obtained
1	39 long days, 0 short days	20	15
2	36 long days, 3 short days	0	10
3	28 long days, 10 short days	0	13
4	21 long days, 17 short days	23	17
5	14 long days, 24 short days	13	16
6	7 long days, 32 short days	15	13
7	42 short days, 0 long days	88	18
8	35 short days, 9 long days	100	21
9	28 short days, 16 long days	100	17
10	21 short days, 23 long days	79	14
11	14 short days, 31 long days	25	12
12	7 short days, 38 long days	4	23

THE SITE OF PHOTORECEPTION

As its common name implies, the larval stages of *E. brassicae* feed below ground level. The existence of this habit in an insect responding to daylength immediately raises the question of the site of photoreception.

The eggs are laid near the soil surface around the host plant and could therefore receive a light stimulus. However, since the eggs only take about 4 days to hatch in the glasshouse conditions the minor occurrence of diapause in the pupae from plants 11 and 12 shown in Table 1 seems to exclude this possibility.

The larvae on hatching from the eggs move rapidly below ground to the root system of the host plant, where the chance of their receiving a daylength stimulus seems remote (see Baumgartner, 1953). When reared on turnips the light may reach the insects through the tissues of the swollen root (cf. *Grapholitha*, Dickson, 1949). Measurements on the absorption of light by turnip tissue of the material used suggest that on the average only 0·6% of the light incident on the plant surface would have reached a feeding larva.

To investigate the site of photoreception the following experiment was designed. The tops of plant pots, in which turnips had been grown in daylengths of 8 hr., were covered by two thicknesses of opaque black cloth drawn tightly around the upstanding leaf pedicels. In this way the light was prevented from reaching both the soil and the swollen root surface.

Groups of thirty eggs, collected within 12 hr. of being laid, were placed around each of these plants and around each of similar plants without covers. Pairs of plants, one covered except for the leaves and the other with both the soil and the swollen root exposed, were given one of the following light-treatments.

(a) 24 hr. light (11 hr. daylight, supplemented throughout the 24 hr. with a mercury vapour lamp giving 250 f.c.), allowing the plants to grow normally.

(b) 24 hr. light (11 hr. daylight, supplemented by 100 W. tungsten lamp giving about one-tenth of the light of the mercury vapour lamp used in (a)).

(c) 8 hr. daylight and 16 hr. darkness.

(d) 8 hr. daylight and 16 hr. darkness during the time when the larvae were developing on the plants, but prior to the eggs being placed on them, the plants were pretreated for 10 days with 24 hr. light as in treatment (a) above.

Table 2. Comparisons of the proportions of diapausing pupae reared from larvae subjected to different light treatments

Plants	Daylength		Proportion of diapausing pupae* (%)	
	Pretreatment	Treatment	Plants with covers	Plants without covers
Pair (a)	8 hr. day	24 hr. day + (250 f.c.)	22	33
Pair (b)	8 hr. day	24 hr. day + (20 f.c.)	8	19
Pair (c)	8 hr. day	8 hr. day	100	100
Pair (d)	24 hr. day + (250 f.c.)	8 hr. day	93	50

* The numbers of pupae used to obtain these proportions varied from 9 to 25.

The results of this experiment, expressed as the proportions of the pupae that went into diapause, are shown in Table 2. All the insects reared in the short days of treatment (c) went into diapause, whilst those subjected to long days showed significant reductions of the amount of diapause. The presence of the opaque covers made little difference to the proportion of insects responding to a direct long-day stimulus, even when dim lighting was used. This result indicates that the plant was acting as the photoreceptor for the insects feeding on it.

The simplest hypothesis would seem to be that a change in the chemical composition of the plant, associated with the long daylength, is the stimulus detected by the insect feeding on the root. The idea of a change in the composition of the plant would seem to be supported by the results of treatment (d). Here, the pretreatment of the plant with 10 long days gave indications of a change in the plant sufficiently stable to be passed on to insects starting to feed about 4 days after the plant had been returned to a short-day treatment. A pretreatment effect may account for the stronger response to the long-day stimulus when this was given before the short days in the experiment shown in Table 1.

Whilst a daylength-perceiving role of the host plant would appear logical in the timing mechanism of a root-feeding insect, the exclusion of a plant effect in *Diataraxia* (Way & Hopkins, 1950) and in *Metatetranychus* (Lees, 1953) suggests that such a mechanism may be unusual.

SUMMARY

1. The overwintering resting stage of the cabbage rootfly (*Erioischia brassicae*) is a facultative diapause in the pupal instar.
2. The induction of the diapause takes place during larval development and appears to be governed by changes in the daylength operating through the host plant.
3. It is suggested that the daylength stimulus is passed on to the insect by way of a change in the composition of its food, and this hypothesis is supported by the finding that a long-day stimulus remains apparent to feeding insects after the host plant has been removed to short-day treatments.

It is a pleasure to thank Mrs D. D. Salter and Mrs J. Selwyn who maintained the fly culture and records throughout the period of this work.

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RESPIRATION IN THE DESERT LOCUST

I. THE CONTROL OF VENTILATION

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INTRODUCTION

The abdomen of the desert locust, *Schistocerca gregaria* Forskål, makes regular and nearly continual pumping movements which ventilate the larger tracheal trunks. The increase in amplitude and frequency of these movements in response to carbon dioxide or oxygen lack is well known (Krogh, 1941), but the mechanisms by which the reaction is controlled have remained largely unexamined.

Matula (1911) believed that the head of *Aeschna* nymphs contained carbon-dioxide receptors which stimulated ventilation, but the idea of regulation via the head has been discarded by later authors. Fraenkel (1932) described sensitive secondary centres in the thoracic ganglia of *Schistocerca*. Roeder (1953) detected a rhythmical discharge from the isolated ganglia of *Melanoplus* in 5% carbon dioxide, and he suggested that the ganglia themselves might respond to carbon dioxide. Recent observations by Weis-Fogh (1960) on *Schistocerca* in flight have again suggested that control via the head may be important.

The nature of earlier experiments, in which one part of the central nervous system was removed and the remainder tested for carbon-dioxide sensitivity, made it desirable to carry out localized tests on the intact system as far as possible, and the present work was undertaken to re-examine the ventilatory centres and if possible to locate the carbon-dioxide receptors, with this in mind.

MATERIAL, METHODS AND NOMENCLATURE

Material. *Schistocerca gregaria* were supplied as week-old adults by the Anti-Locust Research Centre. They were kept in cages as described by Hunter-Jones (1956), and fed with sprouting wheat shoots. Locusts at all stages of maturity and of both sexes were used in the following experiments.

Methods. Ventilation was recorded on a revolving smoked drum with a light lever attached by a thread to the third abdominal sternum. A second lever attached to the head recorded neck and prothoracic ventilation. This method allows accurate recordings of the frequency and an estimate of changes in amplitude to be made. A more elaborate technique for recording the actual volume of air pumped was considered unnecessary for the present work where only qualitative information was required. The preparation was placed in a Perspex gassing box (capacity

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500 ml.) and the threads to the levers passed through holes in the lid. The box was perfused with mixtures of oxygen, nitrogen and carbon dioxide supplied from three calibrated flowmeters. Analyses of the gas were made periodically to check its composition (Scholander, 1951). Low carbon-dioxide tensions in air give rise to a much more immediate and vigorous ventilatory response than oxygen lack, so they were used as test stimuli in the experiments on the location of the ventilation regulators. Unless otherwise stated low concentrations of carbon dioxide are always in air.

Nerve impulses were recorded by lifting the nerve into air on two hooked platinum and 10% iridium electrodes, insulated down to the hooks with 'Araldite', and mounted on two Zeiss micromanipulators. The following Tektronix equipment was used to amplify and display the recordings: two low-level a.c. preamplifiers, type 122; a plug-in, dual trace, d.c. preamplifier, type 53/54C; a cathode ray oscilloscope, type 532. Photographic recordings were made with a Shackman camera, AC 2/25.

Respiratory movements were recorded on the oscilloscope by means of a manually operated tapping key which substituted small high-frequency pulses for the 50 cyc./sec. wave of the time marker ('buzzer').

Experiments were carried out at 18–20° C.: in a second series the temperature was raised to 28–30° C., but the results obtained were similar.

Nomenclature. Muscles are numbered according to the scheme used by Snodgrass (1935). The spiracles are numbered 1–10 from the anterior, regardless of their position on the thorax or abdomen. The separate ganglia of the abdomen are numbered 1–5; it should be remembered that the metathoracic ganglion is fused with the first three abdominal ganglia.

VENTILATION MOVEMENTS

Four types of rhythmical ventilation movement make their appearance in the non-flying locust:

(1) Active raising and lowering of the abdominal sterna by the primary respiratory muscles (Snodgrass, 1935). This represents type 2 in the scheme of Plateau (1884).

(2) Longitudinal telescoping movements of the abdominal segments by the secondary respiratory muscles. Both movements are again active.

(3) Protraction and retraction of the head, which I have termed 'neck ventilation' (Fig. 1). Retraction, in phase with abdominal expiration, is brought about by the contraction of muscles 49, 54 and 57, while protraction (inspiration) is by muscles 50, 51, 52 and 53 which straighten the cervical sclerites.

(4) Protraction and retraction of the prothorax, which is termed 'prothoracic ventilation'. Retraction is brought about by the contraction of muscles 59 and 60, and protraction results from the elastic return of the ventral parts of the pronotal flanges, which during expiration ride over the anterior part of the mesothorax (Fig. 1).

Du Buisson (1924) noted neck ventilation in *Stenobothrus* and, prior to flight, in *Melolontha*. It has been observed during the present investigation in the following

additional Acrididae: *Locusta migratoria*, *Anacridium aegyptium*, *Nomadacris septemfasciata* and *Eyprepocnemis plorans*.

The first type of ventilation is more or less continuous in the locust under normal conditions, although subject to great variation in amplitude and frequency. The other types are auxiliary ventilating mechanisms called upon for short periods after great activity: longitudinal telescoping by the abdomen first appears, then neck, and finally prothoracic ventilation.

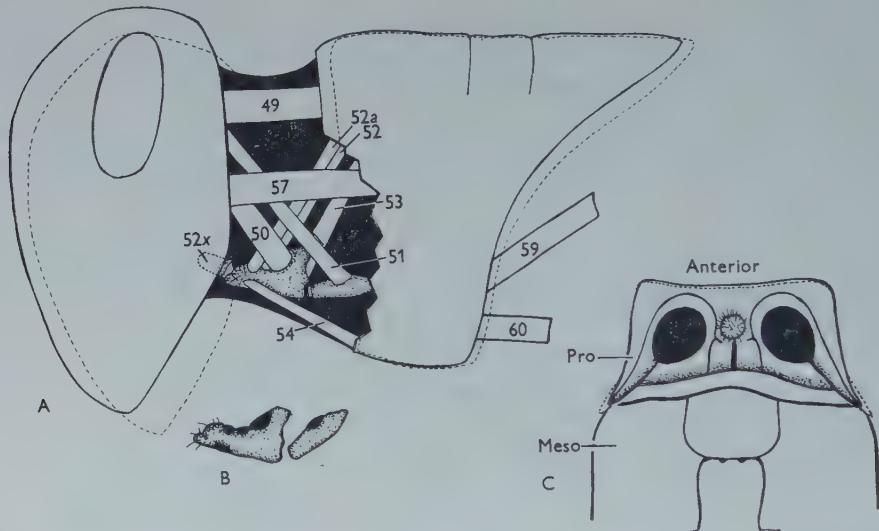


Fig. 1. A, lateral view of the muscles responsible for neck and prothoracic ventilation in the locust. Broken lines indicate positions at the end of expiration. B, the position of the cervical sclerites at the end of expiration. C, ventral view of the pro- and mesothorax showing the outward movements of the pronotal flanges (broken lines) during expiration.

In immature adults, or in locusts kept below 10° C., normal ventilation may be regularly interrupted by pauses when little or no movement occurs. Fig. 2 is a continuous record from a mature locust at 10° C., where approximately 2 min. bursts of ventilation are interrupted by 1 min. pauses.

Measurement of the volume of air pumped by the neck and prothorax

Weis-Fogh (1960) has shown that vigorous abdominal pumping (dorsal-ventral and longitudinal movements) can provide a maximum of 300 l. air/kg./hr. (167 mm.³ per ventilatory stroke for a locust weighing 2 g.). The following measurements were undertaken to determine what extra volume could be pumped by the neck and prothorax.

Spiracles 1 were sealed and the anterior part of the pterothorax was waxed into one end of a tube (5.0 × 1.5 cm.) so that the head and prothorax could move freely inside it. The tube was filled with coloured water to which a very small amount of detergent had been added, and a narrow tube (internal diameter 2 mm.) was corked into the far end. The preparation, with both tubes almost horizontal, was perfused

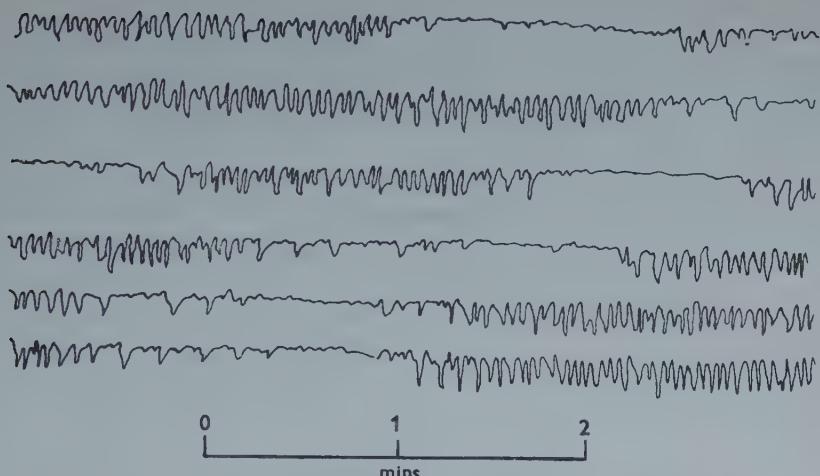


Fig. 2. Tracings of a continuous kymograph record of abdominal ventilation in a resting locust at 10° C., showing six pauses (expiration, upwards).

Table 1. Volume of air pumped by neck and prothoracic ventilation

Locust	Type of ventilation	Frequency of abdominal ventilation/min.	mm. ³ /ventilation	l./kg./hr.
Female wt. 3.2 g.	N and P	30	31	17
	N and P	45	36	30
	N and P	60	45	50
Male wt. 2.5 g.	N	30	18	13
	N and P	30	23	16
	By difference }	30	5	3
	P	60	32	44
	N and P			

Usually at frequencies greater than 60/min. the amplitude diminishes.
N, neck; P, prothoracic.

with carbon dioxide. The resulting neck and prothoracic ventilation moved the meniscus up and down in the narrow tube; the distance it travelled was measured under a binocular microscope. Simultaneous recordings of the abdominal ventilation frequency were made on a kymograph. The volume of water moved in the narrow tube was taken to represent the additional volume of air pumped by the neck and prothorax. Further measurements were made after the removal of the abdomen, but the amplitude of neck and prothoracic ventilation did not alter appreciably. The results from a male and a female locust are shown in Table 1. Altogether twenty locusts have been tested and they have shown that neck and prothoracic ventilation can together contribute a maximum of about 14% of the total volume of air pumped by a non-flying locust, neck ventilation providing 11% and prothoracic ventilation 3%. The significance of these auxiliary forms may be greater than the figures suggest, since they ventilate primarily the head through spiracle 1.

THE ORIGIN OF THE VENTILATORY RHYTHM

It has been known for a long time that each abdominal ganglion can initiate independent ventilating movements in its own segment (Baudelot, 1864), and that one or more thoracic ganglia contain a pacemaker which controls the rhythm in the whole insect (Fraenkel, 1932).

In the following experiments the central nervous system was sectioned at particular sites under carbon-dioxide anaesthesia and the cuticular wounds were sealed with wax. The locusts were observed periodically for several days and in some cases for 3 or 4 weeks after the operation.

After section between the meta- and mesothoracic ganglia all sign of rhythmical movements disappears from the segments anterior to the operation; the synchronized movements of spiracles 3-10 and abdominal ventilation continue with only a slight reduction in amplitude and frequency. If the nerve cord is then sectioned between the metathoracic and the first abdominal ganglia the rhythm persists unchanged in the first three abdominal segments and in spiracles 3-5 (supplied by nerves from the metathoracic ganglion), while it drops to a low frequency and amplitude in the remaining abdominal segments. Likewise, after section between the metathoracic and first abdominal ganglia of an intact locust, the rhythm anterior to the operation persists with little change while that posterior falls to a low level.

The results of sectioning between abdominal ganglia have confirmed that all are able to initiate at least weak pumping movements.

The rhythm initiated by the metathoracic ganglion is nearly always faster and more vigorous than that by the abdominal ganglia, and this ganglion probably contains a pacemaker which drives the slower rhythms of the abdominal ganglia and initiates synchronized movements in the more anterior segments.

Hoyle (1959) describes a rhythm in spiracle 2 of the locust which arises from the isolated mesothoracic ganglion: for reasons discussed elsewhere (Miller, 1960), this rhythm may have no relation to that occurring in the intact insect. There appears to be no rhythmical centre anterior to the metathorax, and after section between meso- and metathorax it is impossible to evoke neck or prothoracic ventilation. Hyperventilation by the abdomen then produces passive movements of the head. Moreover, the suboesophageal ganglion must be in communication with the metathoracic for these auxiliary forms to appear.

THE REGULATION OF VENTILATION

Nervous regulation. By nervous regulation is meant an overriding control from higher centres. It is apparent, for example, during handling, when extremely high frequency (180-220/min.) and shallow amplitude ventilation appears, which cannot be evoked by any combination of high carbon dioxide, low oxygen tension and increased temperature. Alternatively, it appears as a complete cessation of ventilatory movements, again during handling or at the start of flight. It has not been observed after decapitation.

Chemical regulation. A number of experiments was undertaken to locate the carbon-dioxide receptors. They provided the following additional observations:

(1) 1% carbon dioxide in air provokes a considerable increase in ventilation frequency and amplitude, while over 10% causes a reduction in frequency and a great increase in amplitude. At lower concentrations much individual variation occurs as to whether hyperventilation is achieved more by increasing the frequency or the amplitude.

(2) Oxygen lack is much less effective in producing hyperventilation than carbon dioxide, 10% oxygen in nitrogen producing approximately the same hyperventilation as 1-2% carbon dioxide.

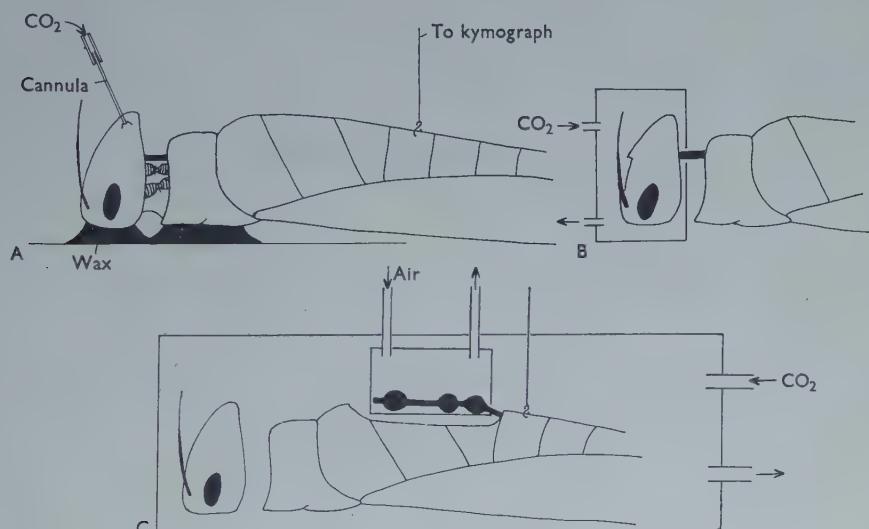


Fig. 3. Techniques for testing the head and parts of the nerve cord for carbon-dioxide sensitivity. A, injection of carbon-dioxide mixtures into the mandibular air-sac through a cannula. The neck tracheae are ligatured and abdominal ventilation is recorded on a kymograph. B, part of the frons is removed and the head placed inside a small gas box. All the neck is cut away except for the nerve cord. C, to test the abdominal ganglia, the thoracic ganglia are perfused with air in a small gas box while the rest of the locust is perfused with carbon dioxide.

(3) In 1% carbon dioxide neck and abdominal longitudinal ventilation are discernible, and in 2% prothoracic movements start.

(4) After 1 hr. in 5% carbon dioxide no type of ventilation shows any diminution as a result of fatigue or sensory adaptation.

Control via the head. To test the head for carbon dioxide sensitivity, a small hole was burnt through the cuticle into the air-sac of each mandible and cannulae were placed in (Fig. 3A). In some experiments all the tissues between the head and the prothorax were removed except for the nerve cord, and a Perspex shield was placed over the open end of the prothorax. Alternatively, the tissues were left intact, but the longitudinal ventral trunks and the tracheae from spiracle 1 to the head were ligatured with fine hair. 0.1-0.5 ml. doses of various gas mixtures were

introduced from a syringe into one cannula and they escaped from the other. Modifications of the ventilatory rhythm were recorded on a kymograph. Since the normal air passages to the head were blocked, it was necessary to inject air into the mandibular air-sacs at frequent intervals.

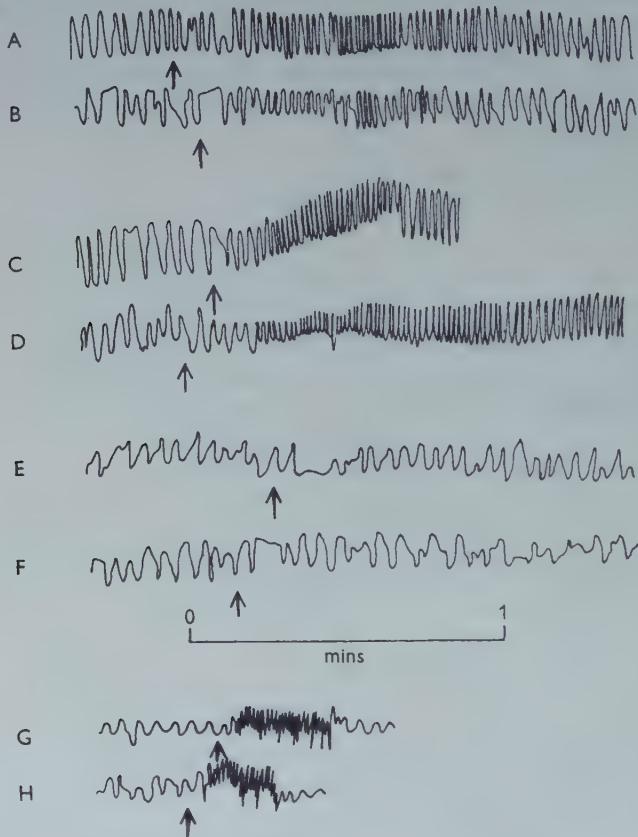


Fig. 4. Tracings of kymograph records of the increase in frequency of abdominal ventilation in response to the injection of carbon-dioxide mixtures into the mandibular air-sac. Arrows mark points at which injections were made. A, 4%; B, 1%; C, 3%; D, 6%; E, air; F, 5% after section of the nerve cord in the neck. G and H, two records of increased abdominal ventilation resulting from perfusing the metathoracic ganglion with 5% carbon dioxide (arrows). Note the faster rhythm superimposed on the slower.

1% carbon dioxide gave rise to a detectable increase in ventilation and more than 4% produced almost immediate hyperventilation (Fig. 4). The injection of air produced either no effect or a decrease in the frequency. After squashing the nerve cords in the neck, similar injections had no effect.

Confirmatory experiments were carried out by cutting out part of the frons, opening the exposed air-sacs and then removing all the neck tissue except for the nerve cord. The head was placed inside a small gas box (capacity 1.5 ml.) and the

nerve cord led out through a nick which was subsequently sealed with petroleum jelly (Fig. 3B). Perfusion of the gas box gave similar results after a slight delay. Cauterization of the supra-oesophageal ganglion did not abolish the reaction, whereas after cauterization of the suboesophageal it disappeared.

The muscles for neck ventilation are innervated from the prothoracic ganglion, and those for prothoracic ventilation from the mesothoracic ganglion. In spite of this it is not possible to evoke either form of ventilation after decapitation. Apparently carbon-dioxide receptors in the head, perhaps the same as those which give rise to abdominal hyperventilation, must be stimulated: they relay to the metathoracic ganglion, which then brings about neck and prothoracic ventilation via the pro- and mesothoracic ganglia.

To conclude, the head contains carbon-dioxide receptors which modify abdominal ventilation. In addition the same or other receptors in the head must be stimulated for neck and prothoracic ventilation to appear. The drop in ventilation which follows decapitation is therefore attributable both to a reduction of the general level of excitation in the central nervous system and to the loss of head receptors.

Control via the thoracic ganglia. Several hours after its complete denervation, except for the posterior connectives, the prothoracic ganglion, attached to part of the longitudinal ventral trachea, was lifted clear of surrounding tissue and placed on a pad of Ringer-moistened filter-paper in the small gas box. The connectives were led out through the nick, which was then sealed with petroleum jelly. The base and sides of the box were positioned with two Zeiss micromanipulators. As with the head, perfusion of the gas box with more than 2% carbon dioxide gave rise to almost immediate hyperventilation which was recorded on a kymograph.

Similar results were obtained from testing the mesothoracic ganglion after removing the prothoracic, and from the metathoracic after removing the mesothoracic ganglion. Further tests, made on each ganglion without removing the more anterior ganglia, gave similar results. They show that each thoracic ganglion can stimulate ventilation when it alone is treated with carbon dioxide, and that this holds true when the nerve cord is intact or after the removal of the more anterior ganglia.

After cutting away the superficial tracheae and air-sacs from each ganglion the response was delayed but not reduced, so that possible receptors must lie on the surface or within the ganglion.

If a gentle stream of carbon dioxide is directed into the open end of the longitudinal ventral trunk attached to the ganglion, or into opened air-sacs on the ganglion surface, hyperventilation follows often in less than 0.5 sec. When the cut ends of the trunk are closed, however, and the air-sacs intact, hyperventilation does not follow in less than 5-10 sec. This suggests that the receptors lie within the ganglion.

At times the faster rhythm induced by carbon dioxide was seen to be superimposed on a slower rhythm (Fig. 4G, H), the latter being due perhaps to the abdominal ganglia alone.

Control via the abdominal ganglia. Tests made on the abdominal ganglia show that in the presence of the thoracic they do not modify ventilation when they alone are treated with carbon dioxide. The tests were carried out by placing all the thoracic ganglia of a decapitated locust in the small gas box and perfusing it with air (Fig. 3C). The preparation, including the small box, was then placed in a larger gas box and perfused with carbon-dioxide mixtures. In this way the whole animal was treated with carbon dioxide, except for the thoracic ganglia. No hyperventilation was produced, and this showed in addition that no gas reached the thoracic ganglia. After removal of the thoracic ganglia, ventilation frequency and amplitude fall to a low level. Carbon dioxide does then cause a slight increase in frequency with occasional ventilations of large amplitude (coughs).

The foregoing tests show that the head and thoracic ganglia are each able to modify ventilation in response to carbon dioxide. (Tests were not made on denervated cephalic ganglia, but by analogy with the thorax it seems probable that the ganglia themselves mediate the reaction.) Stahn (1928) concluded that the receptors which modify ventilation in *Dixippus* are situated in the tracheae close to the spiracles. To test for the presence of additional receptors in *Schistocerca*, small amounts of 10% carbon dioxide were injected from a silicone-lined pipette into various parts of the thoracic tracheal system and the delay before the onset of hyperventilation was measured. Injections into the longitudinal ventral trunks, which supply the ganglia, produce almost immediate hyperventilation; injections into other tracheae produce a response after a longer delay or frequently no response at all. Intravital methylene-blue staining has failed to reveal nerves associated with the longitudinal trunks, and it is probable that all carbon-dioxide reception takes place within the ganglia.

ELECTRONIC RECORDING

Recordings from various parts of the isolated thoracic and abdominal nerve cord have provided further evidence for some of the foregoing conclusions.

Records from any part of the cord anterior to the metathoracic ganglion have failed to demonstrate a rhythmical discharge, although it seems clear that such must exist. Records from the posterior connectives of the metathoracic ganglion or from the lateral nerves to the first three abdominal segments, after they are squashed distally, usually show a rhythmical discharge: it can be seen to correspond to the ventilatory frequency if the lateral nerves are not squashed or if the more anterior ganglia are left intact (Fig. 5). The frequency of impulses and of the rhythm increases after treatment with 5% carbon dioxide.

A rhythmical discharge has been detected in the lateral nerve stumps of each isolated abdominal ganglion. From 10 to 15 bursts occur per minute and the frequency of impulses, although not that of the rhythm, increases in 5% carbon dioxide (Fig. 5). Recording simultaneously from the two lateral nerves of one ganglion shows a similar, but not identical, pattern of impulses.

If the bursts of impulses in fact comprise the motor excitation which normally brings about ventilation, then these recordings show that the bursts are initiated centrally and can be modified in response to carbon dioxide in the absence of any extra-ganglionic sensory mechanism.

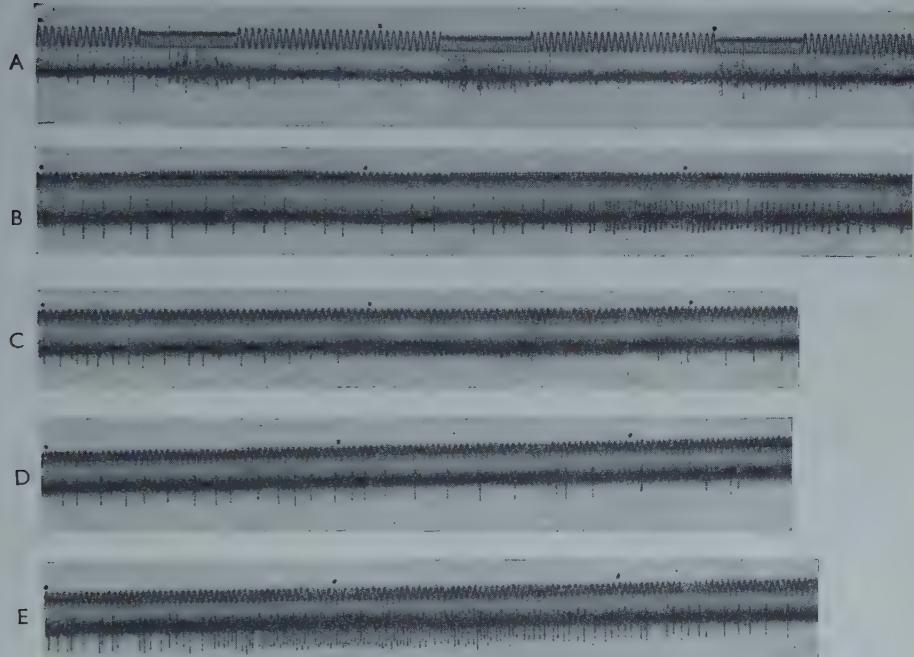


Fig. 5. Oscilloscope records from the connectives of the nerve cord and from the lateral abdominal nerves of the locust. A, connectives posterior to the metathoracic ganglion ('buzzer', abdominal expiration). B, slow rhythm in the lateral nerve of the first abdominal ganglion, after section between it and the metathoracic ganglion ('buzzer', expiration in the second abdominal segment). C, one cycle of a slow rhythm in the lateral nerve of third abdominal ganglion after its complete isolation. D, same from the isolated second abdominal ganglion. E, same after blowing carbon dioxide at the ganglion. Time marker, 50 cyc./sec. (trace); 1·0 sec. (dots).

DISCUSSION

The sites of the centres initiating and regulating the rhythm are summarized in Fig. 6.

The response to partial anoxia has not been investigated in detail, but the evidence suggests that it acts at the same sites as carbon dioxide and produces similar modifications of ventilation. It was pointed out that the effect of 10% oxygen in nitrogen is comparable to that of 1–2% carbon dioxide. Analysis of the composition of the gas in the thoracic air-sacs during flight (Weis-Fogh, 1960) has shown that there is commonly about 5% carbon dioxide and 15% oxygen present. If these values can be taken as representative of the concentrations at the gas-sensitive sites within the ganglia, then carbon dioxide would appear to provide the

more important ventilatory stimulus in the intact insect. Since carbon dioxide diffuses through animal tissues faster than oxygen (Krogh, 1919) such analyses may be misleading. However, assuming that the flight muscles are the prime source of carbon dioxide and users of oxygen during flight, then gas tensions will probably be little different in the ganglia and in the air-sacs.

The occurrence of pauses in the ventilatory rhythm of resting locusts and the occasional failure to detect a rhythmical discharge from the isolated metathoracic ganglion suggest that a low tension of carbon dioxide is necessary to initiate the rhythm. The pauses in ventilation are reminiscent of Cheyne-Stokes ventilation in man and may have a similar origin, namely, the periodic washing out from the blood of chemical stimuli necessary for ventilation.

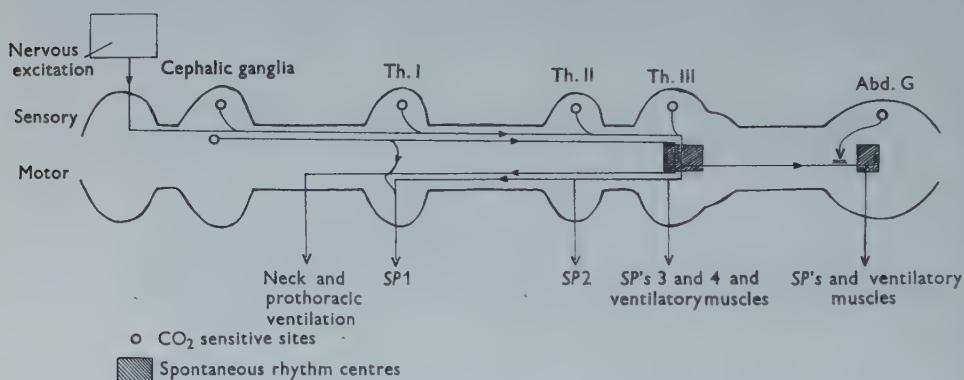


Fig. 6. A summary of the control of ventilation in the locust. Sensory fibres coupled to carbon-dioxide receptors in each ganglion run in the nerve cord to the metathoracic ventilation centre. Other fibres run from the head to the metathoracic ganglion and then to the neck and prothoracic ventilation muscles. Further explanation in the text.

The excitatory effect of carbon dioxide on some nerves and ganglia has been demonstrated by Boistel, Corabœuf & Guérin (1957). This effect may be responsible for the ventilatory response, and possibly carbon dioxide acts directly at the synapses of the motor neurones which supply the ventilatory muscles. Hoyle (1960) has shown that carbon dioxide has a direct effect on the process of neuromuscular transmission in the closer muscle of spiracle 2, reducing the electrical responses and the tension developed. The effect of carbon dioxide on neuromuscular transmission is inhibitory, therefore, compared with the excitatory action on the ganglion. Both effects serve to increase ventilation.

The direct action of carbon dioxide at the spiracle muscle and its possible direct action on the motor neurone synapse both lead to an economy in sensory nerves and perhaps in connexions within the central nervous system. Whether this has any significance for the insect is unknown, but it is worthwhile recalling the small total number of neurones in the insect nervous system, which is available to perform the many observed behaviour patterns (Wiersma, 1952).

SUMMARY

1. Normal (dorso-ventral) and three auxiliary ventilating mechanisms (neck, prothoracic and abdominal longitudinal) are described in the non-flying *Schistocerca gregaria*.
2. Neck and prothoracic ventilation together contribute 14% of the maximum volume of air pumped by the insect. Head ganglion receptors must be stimulated for these forms to appear.
3. The metathoracic ganglion may contain a pacemaker controlling the frequency and amplitude of all forms of ventilation. Each head and thoracic ganglion contains carbon-dioxide receptors which modify the activity of the pacemaker. There is no control from the abdomen in the intact insect, or from receptors outside the central nervous system.
4. Oscilloscope recordings from the isolated central nervous system demonstrate a rhythm, which is modified and possibly initiated by carbon dioxide.
5. It is suggested that carbon dioxide normally provides a more important ventilatory stimulus than oxygen lack.

I would like to thank Prof. V. B. Wigglesworth under whose supervision this work was carried out. I am most grateful to Prof. T. Weis-Fogh for much encouragement and help, and for permission to quote his unpublished results. My thanks are due also to Mr F. Darwin and Mr J. S. Edwards for reading the manuscript. I am grateful to the Agricultural Research Council for financial support.

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RESPIRATION IN THE DESERT LOCUST

II. THE CONTROL OF THE SPIRACLES

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INTRODUCTION

The observations of a number of authors have shown that the spiracles of various insects are capable of graded opening. In the absence of ventilation this is termed diffusion control. Hazelhoff (1927) demonstrated diffusion control in *Periplaneta*: in 1% carbon dioxide the spiracles are slightly open, 2% causes further opening and in 3% they are wide open. It has been observed in the flea (Wigglesworth, 1935), in houseflies (Case, 1957) and in the tsetse fly (Bursell, 1957), and its occurrence has been inferred in the pupae of *Hyalophora* (Buck, 1958), whose spiracles are very slightly open during the 'interburst'.

Graded opening has not previously been demonstrated in an insect whose spiracles are normally synchronized with abdominal ventilation, and in this paper it will be shown that the spiracles of the locust can individually modify their behaviour within the overall pattern of synchronization, and suggestions will be made as to how this is controlled.

The first four pairs of spiracles of the locust open during the inspiratory phase of abdominal ventilation and the remainder during expiration (McArthur, 1929). That these synchronized movements in fact produce an anterior to posterior flow of air through the insect has been demonstrated by Fraenkel (1932). More recent measurements of the volume of air pumped through the insect in flight (Weis-Fogh, 1960) have shown that hyperventilation does not greatly increase the flow, so that probably a modification of spiracular behaviour takes place.

MATERIAL, METHODS AND NOMENCLATURE

Material. Adult *Schistocerca gregaria* were obtained and kept as described elsewhere (Miller, 1960a). The first three pairs of spiracles have been studied in detail, and observations made on the remainder suggest that they are similar to the third.

Methods. To record the behaviour of several spiracles simultaneously, very small mirrors were attached to the spiracle valves with a resin and wax mixture. The mirrors were made from silvered fragments of drawn out coverslips and each weighed approximately 0.05 mg.—about the same weight as one valve of spiracle 2. A weight twenty times heavier did not hamper the movements of the valves. A beam of light from a Baker microscope lamp was reflected by the mirrors through

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concentrating lenses on to a moving roll of photographic recording paper (Kodak R.P. 20), in a simple electrically driven camera. Movements of the spot on the film which resulted from ventilation were clearly distinguishable from those caused by opening and shutting of the valves. Where only the extreme positions of valve movement were required, the beam was focused onto a ground-glass screen and the positions marked by pencil. A slight but negligible error arose from not using a cylindrical screen.

Before a mirror can be attached to spiracle 1, part of the overlying pronotum must be cut away and the prothorax rigidly waxed to the mesothorax. Spiracles 3 and 10 are sunk in pits, and the mirror must be supported on a very small glass rod waxed to the valve. With this technique it was possible to record from three spiracles simultaneously.

Nerve impulses in the spiracular nerves were recorded with two hooked platinum and 10% iridium wire electrodes, 0.03 mm. in diameter, and insulated down to the hooks with 'Araldite'. The nerve was lifted into a drop of paraffin oil contained in a polythene ring (diameter 1 mm.), which was glued to one electrode. Frequently this failed to contain the oil, but it was still possible to record impulses when more was added. Provided that oil did not spread into the cut ends of tracheae, the nerve remained alive for at least an hour. The impulses were amplified, displayed and photographed as described elsewhere (Miller, 1960a). Impulses were recorded from the spiracle nerves close to the ganglia and close to the spiracles but no differences were detected.

Spiracle movements and other events were recorded on the oscilloscope by the 'buzzer' method (Miller, 1960a).

Nerves were stimulated with two coupled, single channel, square-wave stimulators, using pulses of 1 msec. duration at various frequencies. Most stimulation was carried out under oil.

Sections of the spiracles and their muscles were cut after fixation in Carnoy or Baker's formaldehyde-calcium and double embedding by Peterfi's Celloidin paraffin technique (Pantin, 1948). The course of the spiracle nerves was traced in fresh material after supravital staining with methylene blue.

Gassing techniques are described elsewhere (Miller, 1960a).

Nomenclature. Nerves are named according to Ewer's (1953) scheme for *Acanthacris ruficornis*. The muscle numbering is that used by Snodgrass (1929, 1935). The spiracles are numbered 1-10. It should be remembered that spiracle 3, situated on the first abdominal segment, supplies tracheae principally to the thorax, while spiracle 4 does so exclusively.

GENERAL OBSERVATIONS ON THE SPIRACLE RHYTHM

Mirror recordings of the activity of spiracles 1-4 and 10, and direct observations on the remainder, have confirmed the conclusions of earlier authors concerning their synchronization with ventilation. Spiracle 10 has been reported as inspiratory at rest but expiratory in flight (Hamilton, 1937). All the present records have

shown that spiracle 10 opens towards the end of expiration, although when ventilation is weak it may in addition open during inspiration.

At rest, spiracles 3 and 5–9 remain closed, so that air probably enters by spiracles 1, 2 and 4, and leaves by spiracle 10. During greater activity, and normally in mature females, spiracles 5–9 are brought into use, the more posterior first. In some locusts spiracle 3 may open only during and for a short period after flight.

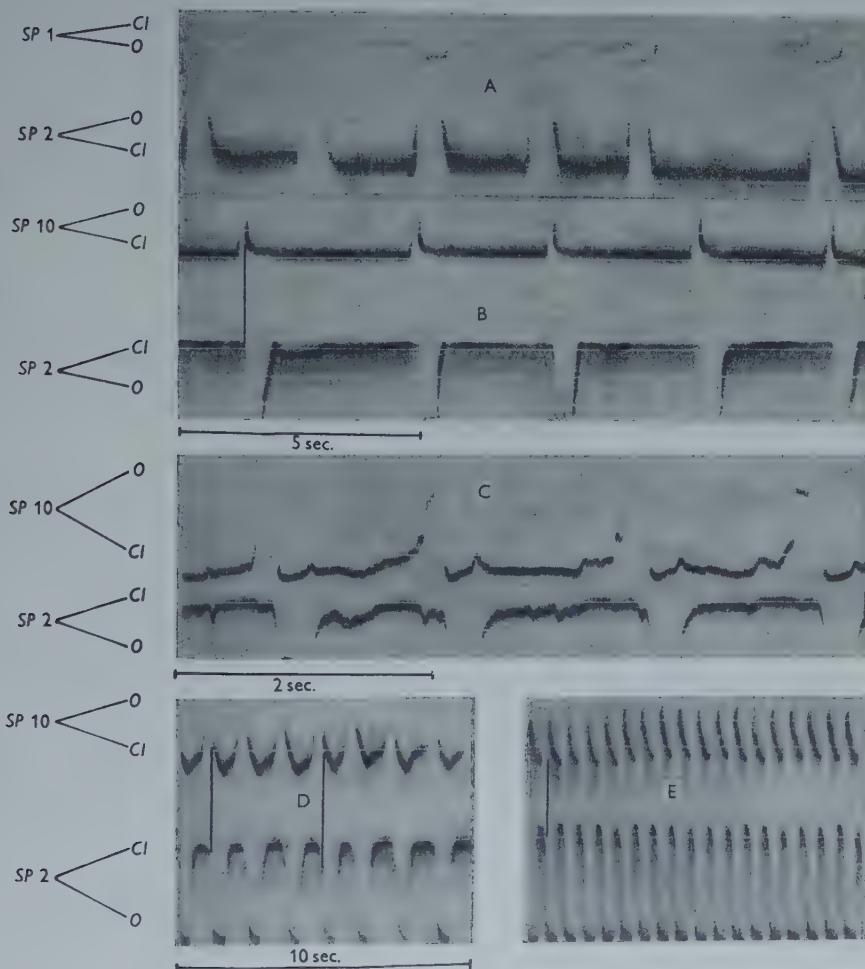


Fig. 1. Mirror recordings of the synchronized movements of pairs of spiracles of the locust. A, spiracles 1 and 2 opening with inspiration. B, spiracles 10 and 2. C, spiracles 10 and 2 in 1% carbon dioxide. D, spiracles 10 and 2 in 3% carbon dioxide. E, spiracles 10 and 2 in 5% carbon dioxide. SP, spiracle; Cl, closed; O, open.

The durations of the open and closed phases of the spiracles are very variable (Hoyle, 1959), but in a resting locust ventilating at 30/min., the open phase of the inspiratory spiracles seldom lasts more than 20%, and that of the expiratory

spiracles 5–10% of the whole ventilatory cycle (Fig. 1). Opening of the inspiratory spiracles always follows that of the expiratory spiracles immediately: there is then a pause, which includes the compression phase (McCutcheon, 1940), before the expiratory spiracles re-open. When ventilation is accelerated, the duration of the closed phase is reduced while that of the open phase stays approximately the same, and the compression phase disappears.

In an atmosphere of 5% carbon dioxide, the inspiratory and expiratory spiracles may each be open for as much as 80–90% of the whole cycle: the considerable overlap allows air to be inspired and expired to some extent through all spiracles. Indirect measurements of the intratracheal pressure of various insects (Watts, 1951) show an increase with moderate ventilation from 1 to 7–10 mm. Hg, whereas in hyperventilation induced by carbon dioxide the pressure falls back to 1–2 mm. These figures are probably explained by changes in spiracular behaviour, similar to those described here.

SPIRACLE 1

Morphology. Adequate general descriptions of the spiracles of Acrididae have been given by Snodgrass (1929, 1935), Jannone (1940), Karandikar (1939) and Albrecht (1953, 1956). Only a brief description and a number of details not mentioned by these authors will be given here.

Spiracle 1 is situated on the soft membrane between the pro- and mesothorax, underneath the posterior expansion of the pronotum, which is kept clear of the spiracle by a small knob. Unlike the other spiracles the atrium leads into two separate tracheal trunks: the dorsal one supplies the head and prothorax and the smaller ventral one the first pair of legs and the flight muscles. The openings of these trunks into the atrium will be termed the dorsal and ventral orifices. The spiracle is contained on a small peritreme to which the anterior grooved valve is fixed and the posterior valve hinged. The ventral part of the posterior valve comprises well sclerotized cuticle and is saucer-shaped; the dorsal part is of soft cuticle with the typical 'thorns' of intersegmental membrane (Fig. 2). A sclerotized rod runs down the anterior margin of the valve; it passes between the orifices and then inwards and round the ventral orifice. Half way round it bears a large process which curves anteriorly and upwards. The closer muscle (79), arising from an apodeme on the peritreme, runs dorsally to this process. One end of the opener (80) shares the insertion on the peritreme with the closer, and the other is attached to the posterior margin of the hinged valve near its ventral end.

The closer muscle is short (0.5×0.3 mm.) with outer fibres $20-30\mu$ in diameter and an inter-Z distance of $3-5\mu$. The central fibres are more slender, $10-15\mu$ in diameter with an inter-Z distance of $5-8\mu$. The opener is longer and thinner (0.15×0.7 mm.) with fibres $10-20\mu$ in diameter and an inter-Z distance of $3-5\mu$.

The spiracle is innervated by the median nerve of the prothoracic ganglion (Fig. 3). The course of the nerve is very similar to that of *Acanthacris ruficornis* (Ewer, 1954a). Near the spiracle it divides and two axons enter each muscle.

As in the cockroach (Case, 1957) and the dragonfly nymph (Zawarzin, 1924), the motor axons themselves divide at the branching of the median nerve into two transverse nerves. This has been shown in two ways; by simultaneously recording from the two transverse nerves close to the spiracles, when identical patterns of impulses are obtained from each (Fig. 4), and secondly, after cutting the median nerve close to the spiracle, by electrically stimulating one transverse nerve when identical movements are seen in both spiracles. Similar tests have shown this to be true for spiracles 2 and 3 as well.

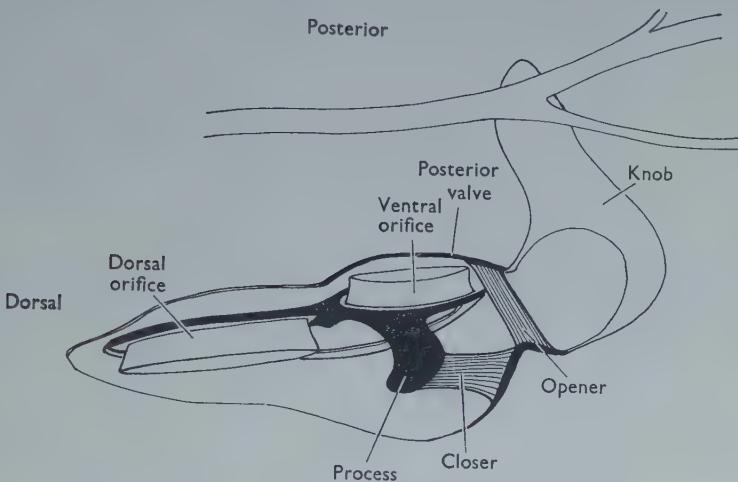


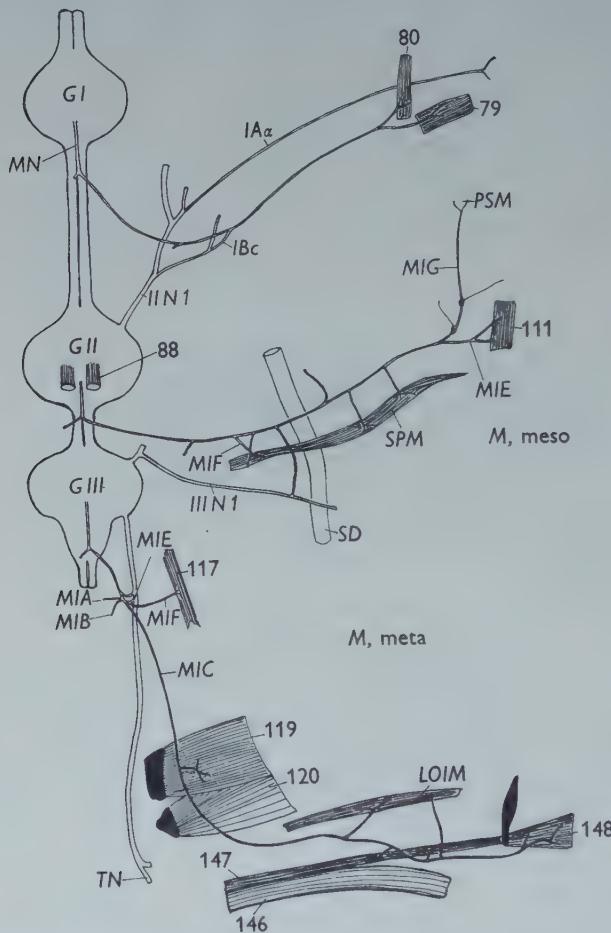
Fig. 2. Spiracle 1, inner view.

Intact action. Mirror recordings were made of the open and closed positions of the valve every 30 sec. in various concentrations of carbon dioxide in air. Fig. 5 shows the average positions from thirty locusts, the vertical bars representing extreme values. In air and when the locust is at rest the valve opens 20–30% with inspiration; it opens wider in increasing carbon dioxide concentrations and maximally in 3–4%. In more than 10% it fails to close fully.

After section of the closer the valve opens 20–30%. Section of both muscles shows that hinge elasticity is responsible for this amount of opening. Wider opening must therefore depend on differential contractions of the opener. By cutting the surrounding cuticle, inverting the spiracle and removing the tracheae, it is possible to watch the activity of both muscles. Together with observations on the intact spiracle this has provided the following account.

In air and at rest the opener makes no contraction, spiracle opening depending on relaxation of the closer and hinge elasticity alone. The action is unaltered after destruction of the opener. In 1% carbon dioxide the valve opens approximately 50% with inspiration and the opener makes rhythmical contractions, which start during and finish after those of the closer (Fig. 6A). The instants of opening and closing of the valve are still determined by the closer relaxations and contractions.

The slower contraction of the opener draws the posterior margin of the moving valve inwards and thereby tenses a cuticular spring. The more the valve is distorted, the more energy is stored by the spring and the wider the valve opens when the closer relaxes. Before the closer contracts again, the opener relaxes and the valve closes from half to about a quarter open. In 2% carbon dioxide the phasing



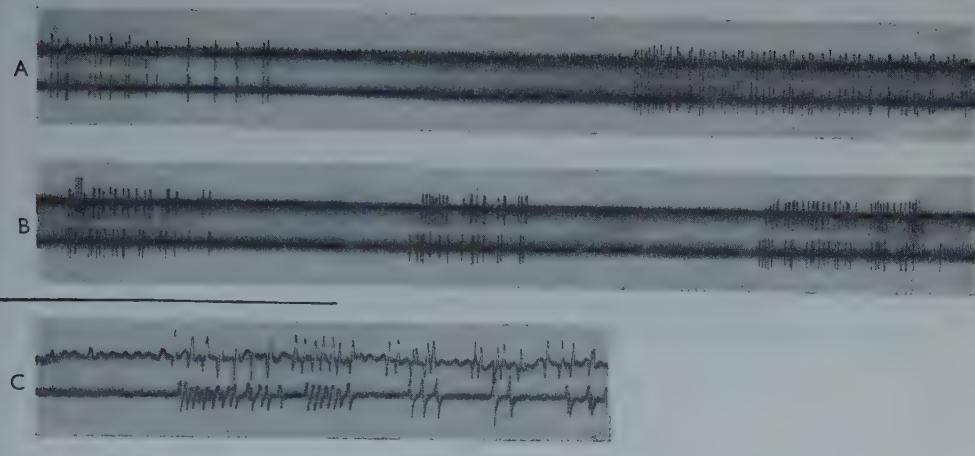


Fig. 4. Oscilloscope records from the transverse nerves of the locust. A, synchronous impulses in the nerves to right and left spiracles 2. B, Simultaneous records from the nerves to spiracles 1 (top) and 2 (bottom). C, simultaneous records from the nerves to spiracles 1 (top) and 3 (bottom).

Horizontal scale for A and B = 1 sec.; for C = 100 msec.

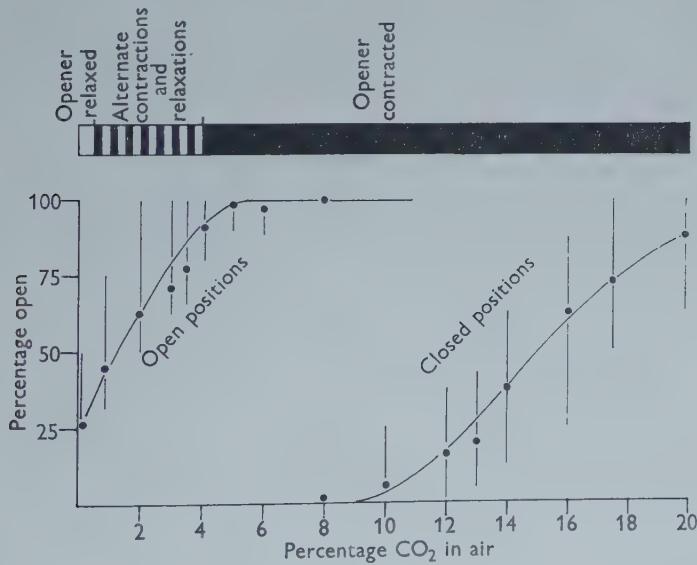


Fig. 5. The percentage opening of spiracle 1 in various carbon dioxide concentrations during inspiration ('open positions') and expiration ('closed positions'). The vertical bars represent extreme values. Above the graph the activity of the opener is represented.

and the spiracle 70% or more open. Finally, in more than 4–5% carbon dioxide the relaxations of the opener get smaller and disappear, so that the opener remains continuously contracted and the closer shuts the valve against the fully tensed spring (Fig. 6C).

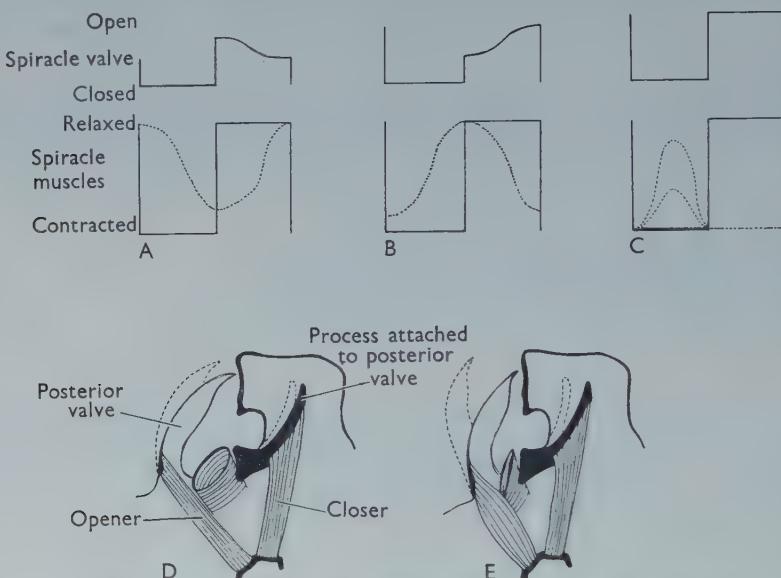


Fig. 6. A–C, the relation of the activity of the closer (continuous line) and the opener (broken line) to spiracle movements. A, in 1% carbon dioxide. B, in 2% carbon dioxide. C, in 4% carbon dioxide; three phases of opener activity are shown. D, transverse section near the ventral end of spiracle 1 illustrating the amount of spiracle opening (broken line) when the opener is relaxed and, E, when the opener is contracted.

Since both muscles make contractions while the spiracle is closed, it is not surprising that both have been termed closers by some authors (Lee, 1925), and some other Orthoptera have been described with two closers in spiracle 1 (Maki, 1938).

A volley of large impulses (up to 80/sec.), recorded from the transverse nerve, corresponds to the contraction of the closer, while a volley of smaller impulses (smaller by a factor of five or six and usually at frequencies lower than 20/sec.) corresponds to the contraction of the opener (Fig. 7G). In air when the opener stays relaxed no small impulses appear, and in 5% carbon dioxide when the opener remains contracted the small impulses are continual. No change in the pattern of impulses has been detected after squashing the nerve peripherally, but after its section near the ganglion they cease. There is no evidence for sensory nerves in the transverse nerve, which are associated with the spiracle. Sensory impulses have been recorded from nerve $IA\alpha$, which probably innervates cuticular sense organs near the spiracle, but they remain unaltered in carbon dioxide.

Inspection of the records reveals that the large (closer) impulses often occur in pairs and are of two sizes; moreover, one usually occurs at a frequency slightly

different from that of the other. This means that they come in and out of phase, that is, they beat (Fig. 7A, B). The same is usually apparent in the small (opener) impulses. When they coincide, the impulses are superimposed and an extra-large impulse is recorded. Similar patterns have been observed in recordings from spiracles 2 and 3.

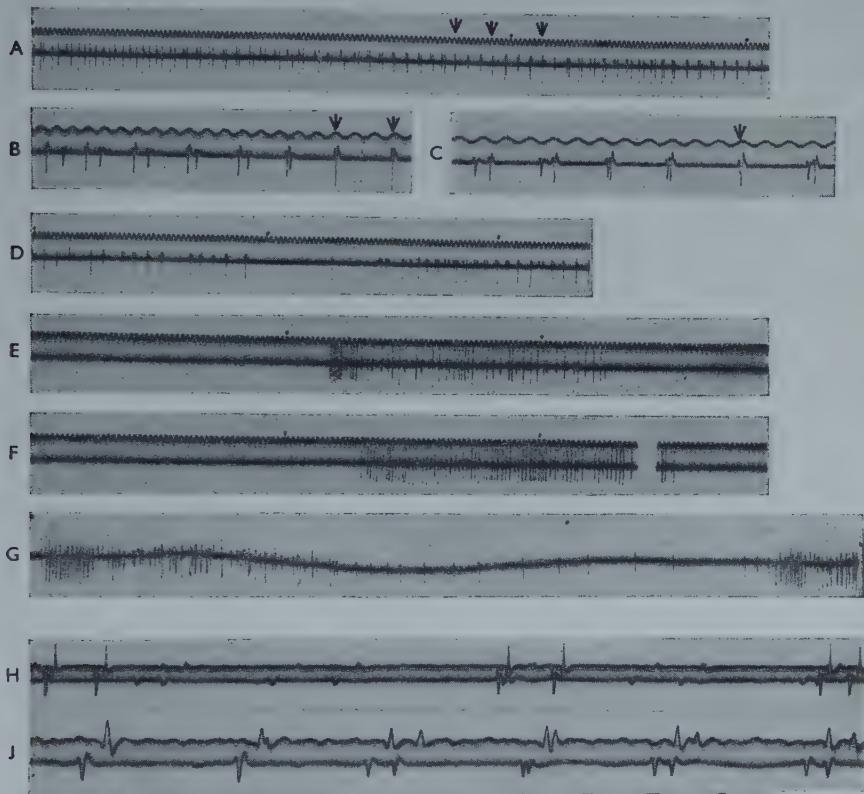


Fig. 7. Oscilloscope records from the transverse nerve to spiracle 1 of an intact locust. A, during a temporary pause in ventilation, closer impulses are maintained and the spiracle remains closed. B and C, same at faster film speed. Arrows indicate extra large impulses resulting from the coincidence of two normal impulses. D, a silent period between two volleys of closer impulses, during which the spiracle opens 20 %. E, small opener impulses appear during and for a short time after volleys of closer impulses, cf. Fig. 6A. F and G, in 5 % carbon dioxide the opener impulses are maintained throughout, cf. Fig. 6C. H and J, simultaneous records from two pairs of electrodes 3 mm. apart on the transverse nerve of spiracle 1. H, closer and opener impulses. J, opener impulses alone. Time markers: A-G, 50 cyc./sec. (trace) and 1.0 sec. (dots); H and J, 20 msec. pips.

Hoyle (1959) describes spiracle 2 of the locust as being innervated by two axons, a 'slow' and a 'fast'. They often fire together but sometimes the slow discharge is absent, while the fast is always there. He has observed pairs of impulses, but states that both members occur in the same axon. However, in spiracle 1, since one member of the pair is of a slightly but constantly different

size from the other, and since the second sometimes follows the first by 1 msec. or less (presumably within the absolute refractory period of the first) and may even be superimposed on it, it seems more likely that each axon provides one member of the pair, and that both axons are therefore involved in the normal operation of the spiracle. Hoyle draws attention to paired impulses in records from the cockroach (Case, 1957); in these, too, extra large impulses occur infrequently.

Measurements of the speed of conduction in the four axons of the spiracle 1 transverse nerve have been made by recording under oil from two pairs of electrodes separated by 3 mm. (Fig. 7H). Impulses in the two axons to the closer travel at approximately 1.09 and 0.97 m./sec.; in the two axons to the opener they travel at approximately 0.43 and 0.36 m./sec. These speeds are slow compared with 6-7 m./sec. in the giant fibres and 2-3 m./sec. in the cercal nerves of the cockroach (Roeder, 1948).

Stimulation of the transverse nerve of spiracle 1 with single shocks, after destruction of the opener, produces twitches in the closer, and these summate to give a smooth tetanus at frequencies greater than 15/sec. The opener, however, does not respond to single shocks; at 3/sec. a very slow and weak contraction occurs; at 6/sec it contracts more strongly still taking 2-3 sec. to do so, and at higher frequencies the contractions become stronger and faster. The artificial and natural frequencies of impulses produce about the same speed and amount of contraction in each muscle, and they show that the closer is a fast (phasic) muscle and the opener a slow and at times a tonic muscle.

The site of spiracle 1 regulation. The foregoing experiments have shown that carbon dioxide affects the activity of the opener. To determine whether this is initiated centrally or peripherally, mirrors were waxed to the valves of both spiracles 1 and a very gentle stream of carbon dioxide directed into one. After a delay of several seconds increased opening occurred in both spiracles simultaneously. By comparison with results from spiracle 2, and since each spiracle receives the same motor input, the reaction probably takes place through the ganglion, rather than by means of a local system. The possibility remains that an extra-ganglionic system may control both spiracles simultaneously. However, after section of the median nerve close to the ganglion the limited amount of spontaneous activity which then appears is not related in the two spiracles (see p. 256).

Modifications in the patterns of nerve impulses in the transverse nerve, which took place as a result of carbon dioxide treatment, were unaltered after crushing the nerve peripherally and after completely denervating the prothoracic ganglion, except for the median nerve and the anterior and posterior connectives. Consequently, carbon dioxide reception must take place within the ganglion or in another segment.

After section of the nerve cord between pro- and mesothoracic ganglia (or between meso- and metathoracic ganglia) all trace of synchronized action disappears from spiracle 1. The valve usually remains 10 or 20% open with continual fluttering movements due to the closer. Prodding the insect, or struggling, causes immediate

full closing, and afterwards the valve opens more widely for a few seconds. In 1-2% carbon dioxide it opens wide and in higher concentrations all fluttering ceases: opening results from a maintained contraction of the opener and relaxation of the closer.

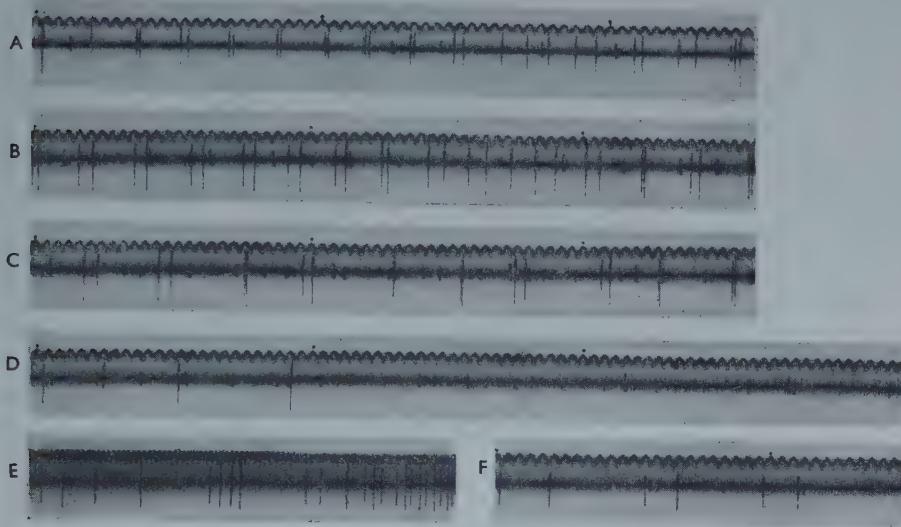


Fig. 8. Oscilloscope records from the transverse nerve to spiracle 1 after section of the nerve cord between the pro- and mesothoracic ganglia. A, in air. B, in 1% carbon dioxide. C, in 2%. D, in 4%. E, touching the antenna ('buzzer') during treatment with 2% carbon dioxide. F, after section of the nerve cord in the neck. Time marker: 50 cyc./sec. (trace) and 0.5 sec. (dots).

Records from the transverse nerve show a constant stream of pairs of large closer impulses (7-12 pairs/sec.)—too slow apparently to maintain a tetanus in the closer (Fig. 8). The slight difference in the frequencies in the axons is more constant than in the intact insect, and the number of extra-large impulses (beats) gives an indication of the frequency difference in the firing of the two axons. With struggling the frequency in both axons increases and the same phasing is maintained. In 1% carbon dioxide a few small (opener) impulses appear but the large impulses are unchanged (Fig. 8B); in 2%, the small impulses increase in frequency and the large decrease; in 3-4% the large disappear altogether (Fig. 8D).

Since section of the nerve cord between the pro- and mesothoracic ganglia abolishes the rhythmic opening and closing movements in spiracle 1, but not its sensitivity to carbon dioxide, the latter must depend on the head or prothoracic ganglion.

Head-perfusion experiments were undertaken to demonstrate a carbon-dioxide receptor which controlled the activity of the opener. (The technique is described elsewhere, Miller, 1960a.) 1-2% carbon dioxide was injected into the mandibular air-sac and it produced almost immediate contraction of the opener. After section

of the nerve cord in the neck the injections had no effect, although 4–5% carbon dioxide directed at the ganglion then produced a weak contraction.

It seems that the head contains sensitive carbon-dioxide receptors whose stimulation increases abdominal ventilation, induces neck and prothoracic ventilation and causes contraction of the opener of spiracle 1. It is possible that one receptor controls all the reactions. In addition the prothoracic ganglion has less sensitive receptors affecting abdominal ventilation and the spiracle 1 opener. Stimulation of the head receptors produces neck and prothoracic ventilation only when the metathoracic ganglion is intact: for opener contractions, however, no more than the prothoracic ganglion need be intact. Since the dorsal trunk leads directly from spiracle 1 to the head, it is not surprising that the head should have an overriding influence on the activity of the spiracle 1 opener. It has no comparable influence over other spiracles.

The ventral orifice. The opener is inserted on the posterior margin of the moving valve behind the ventral orifice, and the closer in front on the upturned process (Fig. 2). When both muscles contract strongly and simultaneously, the long narrow orifice is completely constricted by the scissor-like action of the rod bearing the process and the posterior margin. In more than 4–5% carbon dioxide, when the opener remains contracted, the orifice opens slightly only during inspiration as the closer relaxes. On the other hand, when the opener remains relaxed the orifice is open all the time. The significance of this surprising consequence of opener contractions (a misnomer in the context) is discussed elsewhere (Miller, 1960b).

SPIRACLE 2

Anatomy. A detailed account of the structure of spiracle 2, together with a description of the histology of the closer muscle and its innervation, has been given by Hoyle (1959). Only a small number of additional points will be mentioned here.

Spiracle 2 possesses a single closer muscle (111) and the valves are opened by the elasticity of the cuticular surround. Hoyle points out the occurrence of two types of muscle fibre in the closer: a central core of thin fibres with an inter-Z distance of $6\text{--}7\mu$, and surrounding thicker fibres with an inter-Z distance of $3\text{--}6\mu$. Inner fibres with an inter-Z distance as great as 10μ have been observed during the present investigation. The thin fibres are more numerous than in the closer of spiracle 1, and the inter-Z distance of many is greater. Hoyle suggests that they might be normal fibres whose development has been arrested, but neither Tiegs (1955), Wigglesworth (1956) nor Smith (1958) mentions a stage in fibre development when the striations appear more widely separated than in the mature muscle.

Secretory tissue occurs in the thickness of the anterior valve, and yellow or colourless droplets have been observed hanging inside the valve after rough handling of the insect. Spiracle 2 of *Romalea microptera* froths a black noxious liquid when the insect is irritated, and the secretions in *Schistocerca* may be similar to this.

After section of the closer muscle the valves open and are separated maximally by $0.1\text{--}0.2$ mm. Observations made during flight, however, show the valves then to be separated by $0.4\text{--}0.5$ mm. This wide-opening is achieved by a cuticular device.

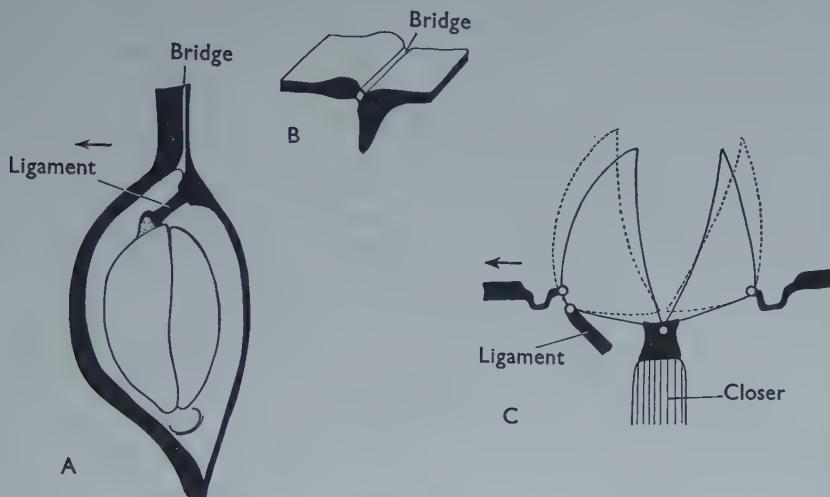


Fig. 9. The wide-opening mechanism in spiracle 2. A, external view showing position of the ligament. B, transverse section of the suture immediately dorsal to the spiracle. C, diagrammatic transverse section of the spiracle. Arrow indicates the movement of the mesothorax. Explanation in text.

The suture between the meso- and metathoracic lateral wall, dorsal to spiracle 2, comprises on the anterior margin of the metepisternum a well-formed apodeme to which the metacoxal muscle (125) is attached. The apodeme is joined to the thickened posterior margin of the mesepimeron by a bridge of cuticle with specific staining properties (Fig. 9B). The bridge, lying under the mesepimeron and barely visible from the exterior, extends about three-quarters of the way from the spiracle to the wings. It is continuous with the cuticle surrounding the spiracle valves but, while the latter acts like a metal spring, the bridge is rubber-like and has many of the properties of the elastic ligaments of the wings (Weis-Fogh, 1958). The rubbery quality is not lost after fixation in alcohol or after drying and re-wetting. In sections fixed in Carnoy or Baker's fixatives and stained with Hansen's haematoxylin or chlorazol black, the bridge stains more intensely than normal cuticle: with Mallory's triple stain it is red.

The bridge allows the mesothorax to be pulled very slightly anteriorly and upwards from the metathorax; on release it snaps back into position. By pulling both ends of a thin horizontal section of the lateral wall under a microscope, the action of the elastic part can be watched. A thin superficial layer of sclerotized cuticle prevents the movement from exceeding about 0.05 mm.

In the nymphs of *Schistocerca* this movement pulls the base of the spiracle valves apart and tends to close the spiracle. In the adult, however, a small and sometimes darkened ligament runs in the cuticle from a knob on the dorsal end of the anterior

valve to the apodeme of the metepisternum (Fig. 9A). It is attached very close but just medial to the vertical axis of rotation of the valve, so that a small anterior movement of the mesothorax pivots the valve wide open on the ligament, and this in turn cant's open the posterior valve (Fig. 9C). After section of the ligament no wide-opening occurs, although the normal movements are unaffected.

Wide-opening has been observed momentarily during struggling and always in flight. It can be artificially induced by gentle pressure on the cuticle just anterior to the spiracle. After section of both pleural apophyses through their respective coxal cavities, it no longer occurs in flight: section of the metathoracic apophysis alone is nearly as effective. Electrical stimulation at 100/sec. of nerve *IVBbi*, which innervates the metathoracic sternopleural muscle (115), causes a slow contraction of that muscle but wide-opening does not occur. Locusts were flown after section of this nerve and of the homologous nerve in the mesothorax, but wide-opening was unaffected. Apparently the rigidity provided by the sternopleural apophysis is essential for wide-opening, but it is not dependent on contraction of the sterno-pleural muscles. Section of the coxal muscles which are inserted on the pleural and intersegmental sutures does not prevent wide-opening in flight, although these muscles are probably responsible for its occurrence during struggling. The basalar and subalar muscles in the mesothorax (98, 99) and in the metathorax (128, 129) assist in the downstroke of the wings (Pringle, 1957), and after their unilateral section, the wing movements of that side are much reduced and wide-opening almost disappears. Comparison with the intact side, where wide-opening continues, suggests that contraction of the basalars and subalars causes it. Electrical stimulation of the mesothoracic basalar and subalar muscles is more effective in producing wide-opening than stimulation of the metathoracic muscles. Weis-Fogh (unpublished) has suggested the presence of tonic components in these muscles, which may be responsible for wide-opening.

Innervation. The spiracle is innervated by the median nerve of the mesothoracic ganglion (Fig. 3). The course of the nerve is similar to that of *Acanthacris ruficornis* (Ewer, 1954a). Near the salivary duct the transverse nerve is joined by a branch from nerve 1 of the metathoracic ganglion. Before and after crossing the duct it supplies a number of branches (meso *IF*) to the vestige of the nymphal spinopleural muscle. Close to the spiracle muscle the transverse nerve divides. One branch, meso *IG* (meso *IE* of Ewer), supplies the vestige of the mesothoracic pleuro-subalar muscle: as it passes dorsal to the spiracle, very slender branches leave it and appear to end on the surface of air-sacs. At the bases of these branches there are swellings in the nerve, which contain large cells. The other branch, meso *IE*, sends two axons into the spiracle muscle.

Intact action. Mirror tests on the open and closed positions of the valves have been made in various carbon-dioxide concentrations (Fig. 10). The valves never open more than 30–40% with inspiration; that is, there is no wide-opening in carbon dioxide. In more than 7–8% carbon dioxide they usually fail to close completely, but this value is variable and in some locusts the valves may make only the weakest closing movements in 5%, whereas in others more or less full closing

continues in 10–12% carbon dioxide. A comparable variation in the sensitivity of the dragonfly spiracles to carbon dioxide has been shown to depend on the water balance of the insect (Miller, unpublished), and the same may be true for the locust. To conclude, spiracle 2 in the non-flying locust shows much less of a graded response than spiracle 1.

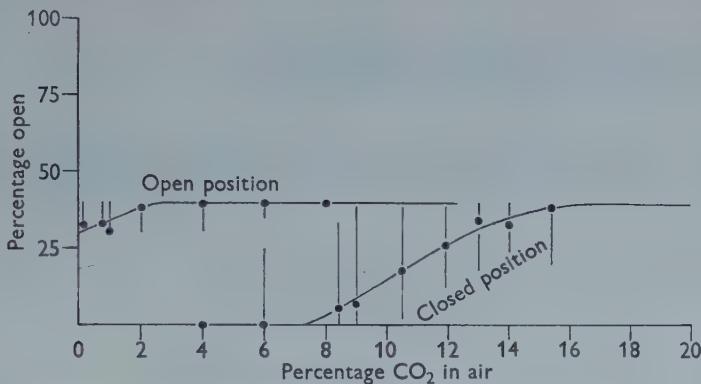


Fig. 10. The percentage opening of spiracle 2 in different carbon dioxide concentrations during inspiration ('open positions') and expiration ('closed positions'). Vertical bars represent extreme values.

Unilateral gassing with 10% carbon dioxide has demonstrated that one spiracle may remain open while its partner continues to make full closing movements: the volleys of impulses remain unchanged in both transverse nerves. The direct action of carbon dioxide on the neuromuscular junction, reducing the electrical responses and the tension developed, has recently been demonstrated (Hoyle, 1960), and is no doubt responsible for the independent action of one spiracle.

The volleys of impulses giving rise to spiracle closing nearly always comprise pairs of impulses of slightly different sizes. For the reasons already given for spiracle 1, both axons appear to be involved in the normal operation of the closer (Fig. 11).

After section of the nerve cord between the meso- and metathoracic ganglia, the behaviour of spiracle 2 is similar to that of spiracle 1. The volleys of impulses in the transverse nerve are replaced by continual pairs of impulses, usually between 6 and 12 pairs/sec. (Fig. 11D). In some locusts the spiracle remains closed, but in many it is 20% open and continually fluttering: with struggling it closes fully. The sensitivity to carbon dioxide is much increased and it will open slightly in 1–2% and completely in 2–3%, when all fluttering ceases. As in the intact insect, the impulses in the transverse nerve remain unaffected during opening. The sensitivity of the response remains undiminished for several weeks.

After the spiracle has been uncoupled from ventilation, the differing frequencies in the two axons become more regular. By watching the valves and listening through earphones to the impulses recorded close to the spiracle, the apparent

drop in frequency as the impulses nearly and then completely coincide can be seen to correspond to a larger twitch of the valve. Periods of coincidence and larger twitches occur about once a second, while there may be 8–10 small twitches per second. Hoyle (1959) describes a rhythm persisting in spiracle 2 after section between meso- and metathoracic ganglia, often seen as a regular rise and fall in the

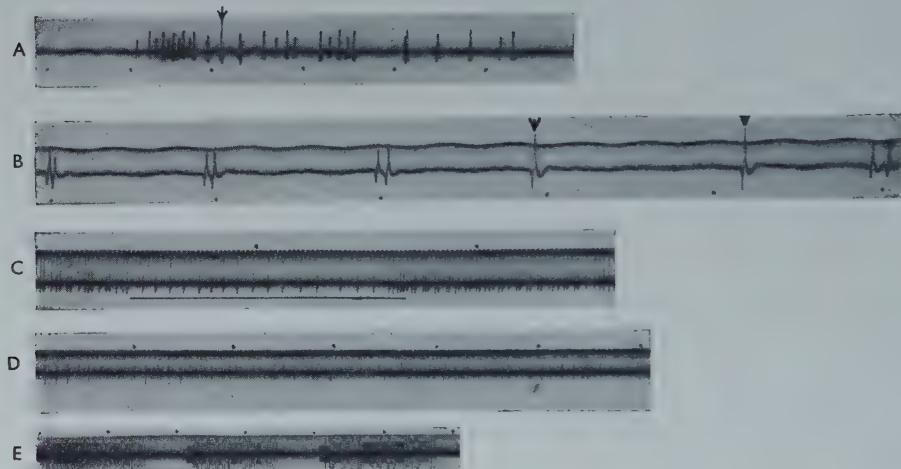


Fig. 11. Oscilloscope records from the transverse nerve to spiracle 2. A, a volley of impulses coinciding with spiracle closing. B, the same at faster film speed. Arrows indicate extra large impulses resulting from the coincidence of two normal impulses. C, during weak ventilation the spiracle opens less than 10% with inspiration and impulses continue at a reduced frequency (horizontal line = inspiration). D, records after section of the nerve cord between meso- and metathoracic ganglia. E, record from an intact locust at low film speed, showing the regular occurrence of extra large impulses. Time markers: A, 100 msec. dots. B, 50 msec. dots. C–E, 50 cyc./sec. and 1·0 sec. dots.

frequency of impulses. The asynchronous firing of the two axons gives the impression of a rise and fall of frequency. This rhythm is clearly of a different nature from that which occurs in the intact insect involving a rhythmic cessation of all impulses. Hoyle states that a pair of impulses separated by 3–4 msec. gives rise to a considerably greater tension than would be evoked by either alone; his account refers to impulses in the same fast axon, whereas the pairs under consideration here, which give rise to larger twitches, occur in different axons.

It is not surprising that the apparent sensitivity of the peripheral carbon-dioxide reaction should be greater after the spiracle is uncoupled from ventilation, since the frequency of motor impulses reaching the spiracle is then less. In an intact but immobilized locust the spiracle may fail to close fully in 7% carbon dioxide, whereas after release full closing may occur in higher concentrations. Variation of the impulse frequency provides a method of altering the sensitivity of the spiracle reaction to carbon dioxide. To what extent it is used, perhaps in relation to the water balance, is unknown.

To conclude, gradations of the open position of spiracle 2 are affected primarily through the wide-opening mechanism and appear only in flight, although a reduced amount of opening (10–20%) is infrequently seen in resting locusts. Gradations of the closed position do not normally take place in less than 5–7% carbon dioxide, and then depend on the local action of carbon dioxide on the neuromuscular junction (Hoyle, 1960).

SPIRACLE 3

Anatomy. Spiracle 3, lying just anterior to the auditory capsule and belonging to the first abdominal segment, supplies large tracheae to the thoracic air-sacs and flight muscles, and also to the gut. The inner end of the atrium is closed by the hinged valve which is produced into a manubrium for the attachment of the dorsal closer and ventral opener muscles (Fig. 12). The short closer, 148 (0.32×0.096 mm.), is inserted on a rigid apodeme immediately above the spiracle, and the long narrow opener, 147 (2.6×0.064 to 0.032 mm.), is inserted on the soft ventral intersegmental membrane, beside the tensor of the tympanum (146). For over half its length the opener runs parallel to muscle 146 and is bound to it by connective tissue. The opener comprises fibres normal in appearance with an inter-Z distance of $3-5\mu$. The structure of the closer is similar to that in spiracle 1. Section of both muscles shows the hinge to possess limited elasticity sufficient to open the spiracle valve 20%.

Innervation. The innervation is again similar to that of *Acanthacris ruficornis* (Ewer, 1953, Fig. 3). The anterior median nerve divides into transverse nerves shortly after leaving dorsally from the metathoracic ganglion. Each transverse nerve (meta IC) sends a branch (meta 1F) to muscle 117 and one to nerve ABD1. It then crosses the ventral end of coxal muscles 119 and 120, sending several branches into the former. Shortly before reaching the spiracle a further branch leaves to supply the vestige of the nymphal longitudinal oblique intersegmental muscle of the metathorax (140). The transverse nerve joins the opener half way along its length and after sending several twigs into that muscle, runs parallel and into the closer.

Intact action. Three patterns of behaviour are commonly seen in spiracle 3 of the non-flying locust: in the first it remains closed, in the second it opens slightly during inspiration and in the third it opens fully.

The first pattern usually appears when the locust is at rest or moderately active and ventilation is not greatly stimulated. At such a time records from the transverse

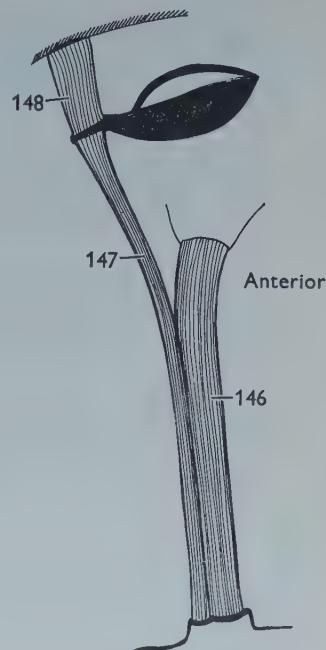


Fig. 12. Spiracle 3, inner view.
148, closer; 147, opener; 146,
tensor of the tympanum.

nerve show a constant stream of impulses (Fig. 13A). The second occurs when ventilation is rapid (*c.* 60/min.) but shallow, and the valve opens about 10% or less in phase with the other inspiratory spiracles: sometimes it opens twice per cycle. Opening results from partial relaxation of the closer and from hinge elasticity; the action is not disturbed by section of the opener. Records from the transverse nerve show alternate volleys of impulses and silent periods (Fig. 13B).

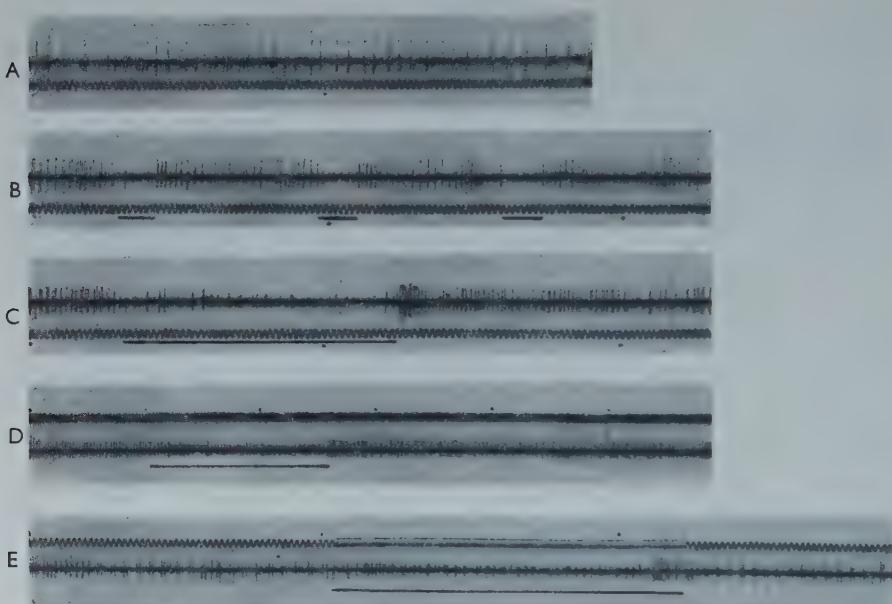


Fig. 13. Oscilloscope records from the transverse nerve to spiracle 3 of an intact locust. A, resting locust; the spiracle remains closed. B, spiracle opens less than 10% with inspiration. C, in 2% carbon dioxide the spiracle opens *c.* 50%, and small impulses appear during opening. D and E, slow ventilation of full amplitude, the spiracle opens fully with inspiration while closer and opener impulses alternate. Horizontal line = spiracle opening. Time marker, 50 cyc./sec. (trace) and 1.0 sec. (dots).

With slow deep ventilation (30/min.), the third pattern appears when the spiracle opens 50–100% during inspiration. The amount of opening is dependent on differential contractions of the opener, although unlike spiracle 1 there is no storing of energy in an elastic system and the contractions of opener and closer alternate regularly. This is reflected in the patterns of nerve impulses from the transverse nerve (Fig. 13C–E), which show a regular alternation of large (closer) and small (opener) impulses—smaller by a factor of 4–5. The small impulses never overlap the large and occur at comparable frequencies (60–70/sec.) when the spiracle opens 100%, and at lower frequencies (10–20/sec.) when the spiracle opens less.

Mirror tests on the open and closed positions of the valve in different carbon dioxide concentrations show that the behaviour of the spiracle is comparable to

that of spiracle 1 (Fig. 14). The amount of spiracular opening is dependent on whether the second or third pattern appears and much individual variation is again seen. In concentrations greater than 10–12% the spiracle fails to close fully, and contractions continue only in the opener, so that the spiracle moves between 20 and 100% open.

Unlike those of spiracle 1, both muscles respond with twitches to single shocks, and tetanus is produced at frequencies of over 15/sec. in both. In the intact insect contractions of the opener are slower than those of the closer.

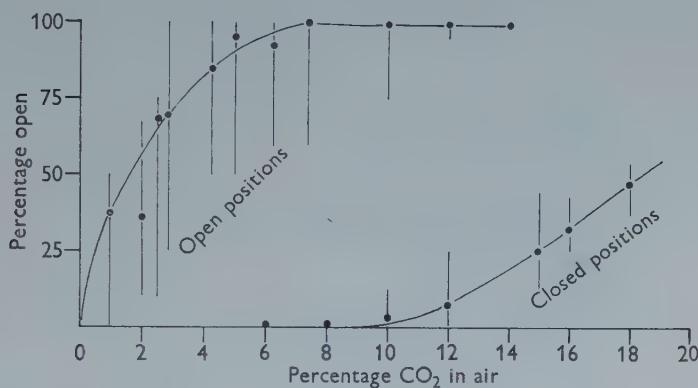


Fig. 14. The percentage opening of spiracle 3 in different carbon-dioxide concentrations during inspiration ('open positions') and expiration ('closed positions'). Vertical bars represent extreme values.

With mirrors fixed to both spiracles and a gentle stream of carbon dioxide aimed into one, it can be seen that modifications of behaviour always affect both spiracles simultaneously. After the destruction of all lateral nerves and the anterior or posterior connectives of the metathoracic ganglion, and after squashing the transverse nerve peripherally, comparable changes in the patterns of impulses with carbon dioxide can still be recorded. It can be concluded that the modifications of spiracle behaviour are regulated entirely from the metathoracic ganglion and do not depend on any external sensory input. Since the ventilatory rhythm originates from the metathoracic ganglion (Miller, 1960a) no method of uncoupling this spiracle from ventilation has been discovered.

As in spiracles 1 and 2, the volleys of impulses to the opener and closer each comprise impulses of two different sizes (Fig. 13) and for the reasons already discussed it is probable that the operation of each muscle is controlled by impulses in two axons.

OBSERVATIONS ON THE DENERVATED SPIRACLES

The continued activity of the denervated spiracle as an independent effector has been described by many authors (Wigglesworth, 1935, 1941; Beckel & Schneiderman, 1956; Case, 1957).

Spiracles 1 and 3. After section of the transverse nerve of spiracle 1 or 3, the opener and closer muscles show no activity. One or two hours later the closer contracts and the spiracle is closed. Subsequently, it relaxes in more than 5% carbon dioxide, but the opener makes no contractions.

Spiracle 2. The left transverse nerve of sixty locusts was cut close to the spiracle, but medial to its junction with meso IG. In the majority the closer showed no more activity for 1 or 2 days. Later it contracted and the spiracle closed. The locusts were kept in a cage under normal conditions (see Part I) and were inspected three times a day. At each inspection the locusts were pinned on one side to a cork and observed for 5 or 10 min. During closing, five stages can be recognized spread over about 24 hr.:

(i) Valves are open and motionless, but close momentarily after one is pulled out and released.

(ii) Small spontaneous movements appear.

(iii) Valves are half closed and make continual small movements.

(iv) Valves are almost fully closed but still moving.

(v) Valves are fully closed.

Most spiracles close after 2 or 3 days—some take as long as 5.

In a further batch of twelve locusts the transverse nerve was cut close to the ganglion, but the subsequent behaviour was unchanged.

Fig. 15 shows the times of closing: eight spiracles closed immediately after nerve section. When the locusts were kept for 3 hr. in the dark or were pinned down and kept at 4° C., then in about 70% spiracle closing followed nerve section immediately. If the locusts were kept immobilized the spiracles remained closed, but shortly after their release they opened. This suggests that the denervated spiracle is normally kept open for the first day or two after nerve section by the level of metabolic end-products in the insect, and that only after 3 hr. immobilization do these fall sufficiently low for the spiracle to close. Herber & Slifer (1928) concluded that the ventilation frequency of *Melanoplus femur-rubrum* did not reach a true 'resting rate' for at least 1 hr. after the insect was pinned down and then only in the complete absence of struggling. It would follow from the hypothesis that spontaneous closing is normally inhibited in the intact locust by the level of metabolic end-products, so that the spiracle is able to open when the motor impulses cease; but this seems improbable and there is no evidence to suggest that the spontaneous closing mechanism is operative in the innervated muscle.

The subsequent closing of spiracle 2 after 2 or 3 days may result from a decrease in sensitivity to metabolic products or an increase in the excitability of the muscle. That it was not a result of water loss through the open spiracle was shown by keeping locusts in moist and in dry air following nerve section, and observing that spiracle closing occurred after approximately the same interval in each.

The closed denervated spiracle is very sensitive to carbon dioxide, opening fully in 1–2% and reclosing shortly after return to air. It opens a few seconds after the start of struggling or flight. It does not react to oxygen lack, and remains closed

in 100% nitrogen until nearly all movement of the locust has ceased. The movements are always slow and smooth—very different from the fluttering seen in the spiracle uncoupled from ventilation. This behaviour is quite unlike that of the denervated cockroach spiracle 2 (Case, 1957) which closes within 24 hr. of denervation and then will not open in less than 15% carbon dioxide. Closing is followed after 4–6 days by 'fasciculation', which Case associates with the degeneration of the peripheral nerve stump.

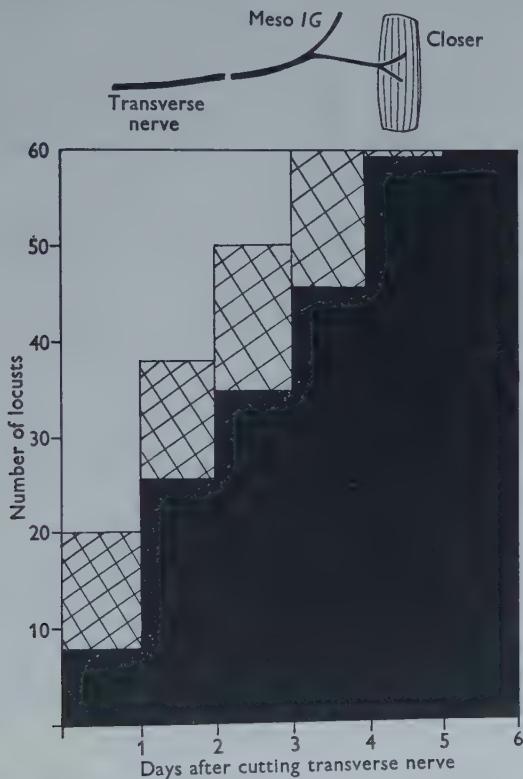


Fig. 15. The time taken by the denervated spiracles 2 of sixty locusts to close. Black area, spiracles at stage 5; hatched area, spiracles at stages 2-3. Eight spiracles close immediately after the operation. Explanation in text.

In the locust the sensitivity remains for 2–3 months; occasionally, when the sensitivity was much reduced, the tracheae supplying the closer muscle were found to be partially filled with liquid. Hoyle (1960) comments on the need for the air passages into the muscle to be unblocked for carbon dioxide to affect the muscle, and this observation suggests that the same mechanism is responsible for muscle relaxation in the denervated spiracle. Nerve regeneration, indicated by the resumption of synchronized movements, occurred in two locusts only (3%). Case (1957) reported regeneration in 36% of operated cockroaches.

It seemed that the independent behaviour of the spiracles could be explained if motor nerve cells were present in the muscle. To investigate this possibility,

6μ sections of the muscle were cut and stained for cholinesterases with myristoyl choline and light green (Denz, 1953; Bowden & Lowy, 1955). Dark areas appeared in the sections but they gave no clear indication of the presence of nerve cells.

The effect of nicotine on insect nervous tissue is well known (Roeder, 1939). After initial excitation, it causes the complete block of ganglia. To test for the presence of nerve cells in the closer, the spiracles were cut out and floated on a solution of 0.001% nicotine sulphate in Ringer. They closed immediately and remained closed for several hours, but would open in 5% carbon dioxide. In stronger nicotine solutions (0.01%) they would not open in less than 20% carbon dioxide. Nicotine excites the closer to contract and its subsequent failure to destroy the autonomous action suggests that contraction of the closer is not dependent on continual excitation from a motor neurone in the muscle, but is a property of the muscle fibres themselves.

Hoyle (1959) pointed out that the thinner muscle fibres with a long inter-Z distance might be expected to perform slow tonic contractions. An attempt was made to destroy most of the large outer fibres with a small scalpel and a tungsten hook. In other spiracles the thin inner fibres, together with the large fibres of one side, were destroyed. The operations were performed on twenty denervated spiracles, and after observation the muscles were fixed in Carnoy and stained with acid fuchsin, so that an estimate of the remaining fibres could be made. Provided that the thin central fibres were not damaged, the spiracle remained closed and continued to react to carbon dioxide. If, however, they were destroyed, but more than half of the large fibres were left intact, the spiracle opened and showed no further activity.

Although not conclusive, the results suggest that the maintained contraction of the denervated spiracle and the subsequent relaxations in low carbon-dioxide concentrations depend on the small central fibres. The observation that the closers of spiracles 1 and 3, possess a few thin fibres and make maintained contractions accords with this suggestion.

DISCUSSION

The operation of the first three pairs of spiracles can be compared when their valves are represented by levers moved by springs and muscles (Fig. 16). This emphasizes the similarity of the closing and differences in the opening mechanisms, the latter being effected in spiracle 1 by hinge elasticity and a muscle in series with a spring; in spiracle 2 by a spring with variable tension (the piston), and in spiracle 3 and the remaining spiracles by a muscle which probably includes a variable elastic element and at times works like a rubber band (Miller, 1960b).

It is noteworthy that the median nerves in each segment, in addition to innervating the spiracles, supply branches to some of the nymphal muscles which degenerate in the adult (Ewer, 1954b). Thomas (1954) suggested that these muscles may make nymphal respiratory movements or possibly that they help to rupture the cuticle at ecdysis. Ewer (1954b) has proposed that they may be important in preserving the shape of the pterothorax immediately after moulting.

Recordings made from meso IG of immature adults, which supplies the pleuro-subalar muscle, have shown bursts of motor impulses which occur during abdominal expiration. The frequency of impulses increases when ventilation is more vigorous. This observation supports both Thomas's first and Ewer's suggestions; however, the situation of the muscles makes it unlikely that they could contribute a significant ventilating movement, whereas their contraction during expiration when the soft pterothorax tends to be blown up, may be important in preserving its shape.

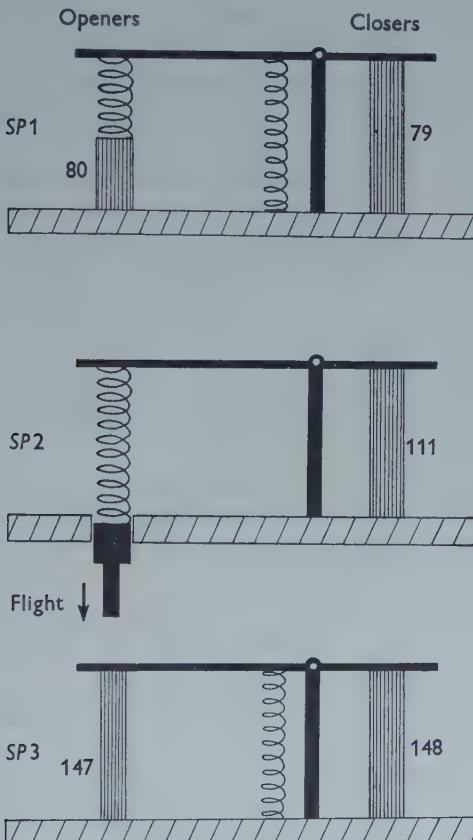


Fig. 16. Models to illustrate the action of spiracles 1, 2 and 3. The spiracle valve is represented by a pivoted lever with the closers on the right and the various opening devices on the left.

It has been shown that the openers of spiracles 1 and 3 are controlled from their respective ganglia and that their contractions in response to carbon dioxide do not depend on receptors outside the central nervous system. In another paper (Miller, 1960a) the possible direct action of carbon dioxide on the membrane of post-synaptic fibres was discussed in relation to the motor neurones of the ventilatory muscles. It seems that a similar mechanism could give rise to the increased activity of the opener motor neurones in response to carbon dioxide, and thereby contribute

further to the economy of interconnexions in the central nervous system, as was suggested elsewhere (Miller, 1960a).

Spiracle 2 is an 'odd-man-out' and it is difficult to explain why this spiracle alone in the locust should have one muscle and a peripheral control mechanism. Observations on the one-muscle spiracles of Diptera and Odonata have shown that they have a similar peripheral control, and this may be a property of all such spiracles. No insect is known to have an opener muscle in spiracle 2 (Maki, 1938), and openers are not found in spiracle 1 outside the Orthoptera. It may be that the peripheral mechanism has some particular significance for the locomotory segments, although what part it plays in the adult locust remains uncertain. Alternatively, the peripheral mechanism may represent a more primitive system which is overridden in the locust in the interests of synchronization with ventilation. There is plenty of evidence (to be discussed in a later paper), however, that in Odonata and Diptera it takes part in the normal control of the spiracle. In the first instar locust spiracle 2 is rarely synchronized with ventilation; more often it appears to act independently and most probably under the influence of the peripheral mechanism.

Both the ventilatory and the spiracular rhythms are autonomous although modified by sensory input (Hoyle, 1959). The presence of carbon dioxide in the ganglion may be necessary for their initiation, but each cycle is not dependent on the build-up and release of the gas. A comparison may be made with the cockroach where ventilation and synchronized movements appear only in more than 10% carbon dioxide (Hazelhoff, 1927); below this the spiracles are regulated perhaps by a peripheral mechanism, similar to that of the locust. The locust, possessing the same mechanisms, is much more strongly biased in favour of ventilation synchronization.

Attention has been drawn to the firing of two motor axons at slightly different frequencies during the normal operation of the spiracle. This has suggested an hypothetical mechanism to account for the rhythmical disappearance of motor impulses in the nerves to the spiracle muscles during ventilation, as follows. Two internuncial neurones each firing spontaneously and continually, but at slightly different frequencies, relay onto the motor neurones of the spiracle nerves. One internuncial is inhibitory and the other excitatory. The excitatory impulse is inhibited at the synapse if, say, the inhibitory impulse precedes it by less than 200 msec. For peripheral α -inhibition in Crustacea the inhibitory impulse must precede the excitatory by only a few msec. to be effective (Katz, 1949); however, at least in vertebrates, a central inhibitory volley can still be effective after 100 msec. (Sherrington, 1947). If, for example, the excitatory internuncial in the locust fires at 10/sec, and the inhibitory at 11/sec., two excitatory impulses will be inhibited at the synapse every second. If two controlling cells are now postulated, one influencing the frequency in both internuncials by the same amount, and the other altering the frequency in one only, both changes in frequency and variations in the duration of periods of firing and of silence can be achieved (Fig. 17). To obtain some combinations it may be necessary to postulate a second inhibitory internuncial.

By some such plan two continually firing cells can produce a much slower rhythm in a post-synaptic fibre without any sensory feed-back. Such a system could be responsible for the rhythmic firing of the motor cells of the ventilatory muscles as well as those of the spiracle muscles.

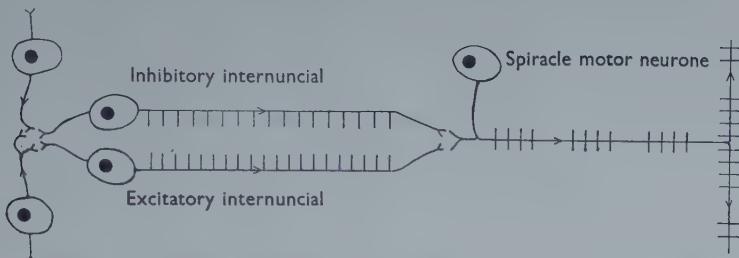


Fig. 17. Diagram to illustrate the theory of the means of producing the ventilatory rhythm.
Explanation in text.

The same principle is used in beat-frequency electronic oscillators, where two oscillators produce a third frequency much lower than that of either alone.

After section of the nerve cord between the meso- and metathoracic ganglia, the rhythm disappears from spiracles 1 and 2, and is replaced by continual firing in the motor nerves. This can be explained if the inhibitory internuncial is in the metathoracic and the excitatory in the mesothoracic ganglion. During strong flight, motor impulses in the nerve to spiracle 2 cease entirely (Miller 1960b); this is explicable if at such times the excitatory and inhibitory internuncials fire in phase.

It should be emphasized that this scheme is entirely hypothetical, and recalled that the patterns of impulses in the transverse nerves involve probably two motor fibres, so that the scheme must perhaps be duplicated.

SUMMARY

- Small mirrors are used to record the movements of the spiracle valves of *Schistocerca gregaria*, and some general observations are made on the synchronized movements of the spiracles with ventilation.

- Spiracles 1-3 are shown to alter their positions of opening and closing in different carbon dioxide concentrations, within the pattern of synchronized movements.

- Modifications of the amount of opening of spiracles 1 and 3 take place as a result of differential contractions of the openers. In spiracle 1 the energy is stored in an elastic system, and the opener does not necessarily make contractions every cycle. Oscilloscope recordings show these reactions, resulting from carbon-dioxide stimulation, are controlled entirely from within the central nervous system and do not depend on any sensory input.

- The movements of spiracle 2 are controlled peripherally through the direct action of carbon dioxide on the muscle membrane (Hoyle, 1960) and also through a cuticular wide-opening device.

5. The maintained sensitivity of spiracle 1 and the increased sensitivity of spiracle 2 after they are uncoupled from ventilation are discussed.

6. Some remarks are made on the activity of the denervated spiracle 2, and the suggestion is made that it depends on a central core of thin fibres.

7. A central nervous mechanism is postulated which could account for the rhythmic cessation of impulses in spiracle and ventilatory nerves.

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RESPIRATION IN THE DESERT LOCUST

III. VENTILATION AND THE SPIRACLES DURING FLIGHT

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INTRODUCTION

The possibility that the thoracic tracheae might be ventilated by flight movements has been realized for a long time (du Buisson, 1924), and Krogh & Zeuthen (1941) have stated that 'abdominal pumping movements and increased ventilation are insufficient during flight and serve mainly to raise the body temperature', and again Krogh (1941) that 'the conclusion appears inevitable that the flight movements must themselves provide the necessary ventilation of the flight muscles'. Not until the work of Krogh & Weis-Fogh (1951), however, was thoracic ventilation more than an inference. These authors were able to show that the resting oxygen consumption of *Schistocerca gregaria* is about 0.63 l./kg./hr., and that during flight it rises to 15 l. The volume of air ventilated by the abdomen at rest is about 40 l./kg./hr. and with very vigorous pumping, induced by carbon dioxide and oxygen lack, a maximum of 300 l. is attained (Weis-Fogh, 1960). However, Weis-Fogh has shown that abdominal ventilation is not pumping more than 180 l. during the first 5 min. of flight and subsequently about 150 l. Consequently, while the oxygen consumption is increased twenty-four times, the volume pumped by the abdomen is no more than four or five times greater.

Thoracic movements during flight account for 600 l./kg./hr., of which 250 l. supply the large anterior abdominal air-sacs and 350 l. the pterothorax. Only the latter is available for pterothoracic respiration, but it is more than adequate (Weis-Fogh, 1953).

Ventilation induced by wing movements can be divided into two steps:

- (i) Ventilation of the intramuscular air-sacs by contraction of the flight muscles.
- (ii) Ventilation of the large extramuscular air-sacs by alteration of the volume of the pterothorax due to vertical movements of the nota and lateral movements of the walls, with each wing-stroke (Weis-Fogh, 1953).

Weis-Fogh has inferred from his measurements of the changes in thoracic volume and pressure that the thoracic spiracles must remain open in flight, but Fraenkel (1932) reported that while in *Libellula*, *Vespa* and *Tipula* they opened immediately flight started and stayed open, in *Schistocerca* they continued to be synchronized with abdominal ventilation. In this paper a re-examination of the behaviour of the spiracles and ventilation during flight is described. It has shown

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that spiracles 2 and 3 are more or less wide open during flight, while the remainder continue to be synchronized with abdominal ventilation. Some suggestions are made as to how this uncoupling is achieved, and the significance of the continued synchronization of the remainder is discussed with reference to modifications of the thoracic tracheal system.

MATERIAL AND METHODS

Material. Adult *Schistocerca gregaria* were used. Arrangements for keeping them have been described elsewhere (Miller, 1960a).

Mature males are the best fliers. Even among these, however, it was necessary to conduct preliminary flight trials on a multiple stand, on which seven locusts could be flown simultaneously in front of the wind tunnel.

Methods. Standard conditions for flying tethered locusts have been described by Weis-Fogh (1956a) and these were followed closely, the wind tunnel used being that described in his paper. The suspension was a simple version of Weis-Fogh's type 3, the locust being fixed by the sterna to a rigid post with a mixture of wax and resin.

24 hr. before flying, the metathoracic and certain other legs, according to which spiracles were to be observed, were removed under carbon-dioxide narcotization and the wounds sealed with wax.

Locusts were flown for up to 2 hr. in a constant wind speed of 3·5 m./sec., at a temperature of 28° C., and with a body angle of 10° to the horizontal. Wing-beat frequency and the flight performance were observed by means of a stroboscope (Stroboflash—Dawe Instrum. Co.).

Mirrors were fixed to the spiracles, and the movements of a reflected beam of light were photographed as described elsewhere (Miller, 1960b). Ventilation frequency was recorded manually with a signal marker on a revolving smoked drum; ventilation amplitude was estimated with the help of a binocular and a scale fixed behind the abdomen.

Weis-Fogh (1956b) has briefly described a method of calculating the metabolic rate of a flying locust from measurements of the steady-state temperature within the thorax, the surface index of the locust (Weis-Fogh, 1952), and the wind speed. To measure the thoracic temperature in flight, a thermistor was mounted in a glass capillary, surrounded by narrow gauge polythene tubing and inserted between the crop and muscle 113. It was waxed in position several hours before flight.

To record impulses from the spiracle nerves during flight, the same electrodes and recording apparatus were used as are described elsewhere (Miller, 1960b). The locust was mounted on the flight stand, which was then turned through 90° so that the spiracle under investigation was uppermost. Impulses were recorded from the nerve under oil. It was not always possible to avoid recording muscle potentials as well, but they are clearly distinguishable on the trace, and give some indication of the wing-beat frequency. When recording from the transverse nerve to spiracle 3, it was necessary to remove most of the hind wing on that side.

RESULTS

During the initial 5 min. of flight the wing-beat frequency is high. After about 10 min. the locust settles down to a steady performance which may continue for several hours with little change. Considerable attention was paid to this initial period as well as to the subsequent steady flight.

Ventilation. For the first few seconds of flight abdominal ventilation ceases entirely. Subsequently, it is resumed at a high frequency and a slightly greater amplitude. None of the three auxiliary ventilating mechanisms (Miller, 1960a) appears during normal flight. If spiracles 1, 2 or 3 are blocked, however, longitudinal abdominal and neck ventilation may continue indefinitely in flight. Blocking more than one pair causes vigorous hyperventilation and a rapid decline of the flight performance. Injection of 0.1 ml. 5% carbon dioxide into the mandibular air-sac causes hyperventilation and the momentary appearance of all auxiliary forms. Injection of larger amounts of 10% carbon dioxide causes excessive hyperventilation and flight soon ceases.

If flight is forcibly stopped during the first 5 min. considerable hyperventilation follows, often involving all three forms of auxiliary ventilation. After longer flights, the ensuing hyperventilation is less marked. These transient increases do not last for more than a few seconds, and must not be confused with the prolonged rise in oxygen consumption lasting for more than an hour after flight (Krogh & Weis-Fogh, 1951).

Locusts were flown for at least an hour after section of the nerve cord posterior to the metathoracic ganglion, and also after complete removal of the abdomen, the wound being sealed with wax. In neither case did the lack of abdominal ventilation appear to affect the flight performance, although neck and prothoracic ventilation continued during and after flight. Apparently abdominal ventilation is not essential for flight.

Spiracle 1. In order to attach a mirror to this spiracle, part of the overlying cuticle was removed and the prothorax rigidly waxed to the mesothorax. This did not reduce the flight performance or impede the spiracle movements.

During the initial few seconds of flight, when ventilation ceases, the valve remains closed but the opener contracts fully (Miller, 1960b). As soon as ventilation starts, the spiracle resumes normal synchronized movements, and the amount of opening is reduced after a minute (Fig. 1). When flight is stopped, the spiracle again opens wide during the first few inspirations, although this is less marked after longer flights.

Spiracle 2. Immediately flight starts, spiracle 2 opens wide and remains open without movement (Fig. 1). The wide-opening mechanism is fully effective. In most locusts incipient closing movements, in phase with abdominal expiration, appear after 5 or 10 min. They normally result in a 10–20% reduction in opening, although when flight is weak and the wing-beat frequency low, they may more than half close the valve. On the cessation of flight the valve immediately closes fully, and then resumes normal synchronized movements. Fig. 2 shows a tracing of a mirror recording from this spiracle.

Appropriate cuts in the cuticle close to the spiracle have been made to put the wide-opening mechanism out of action. During flight the spiracle remains 30–40% open and no movement occurs in the valve for the first few minutes. This shows that the closer muscle is not prevented from closing the spiracle by the wide-opening mechanism, but initially makes no contraction, and subsequently only very weak contractions. It has been confirmed by lightly pressing the cuticle close

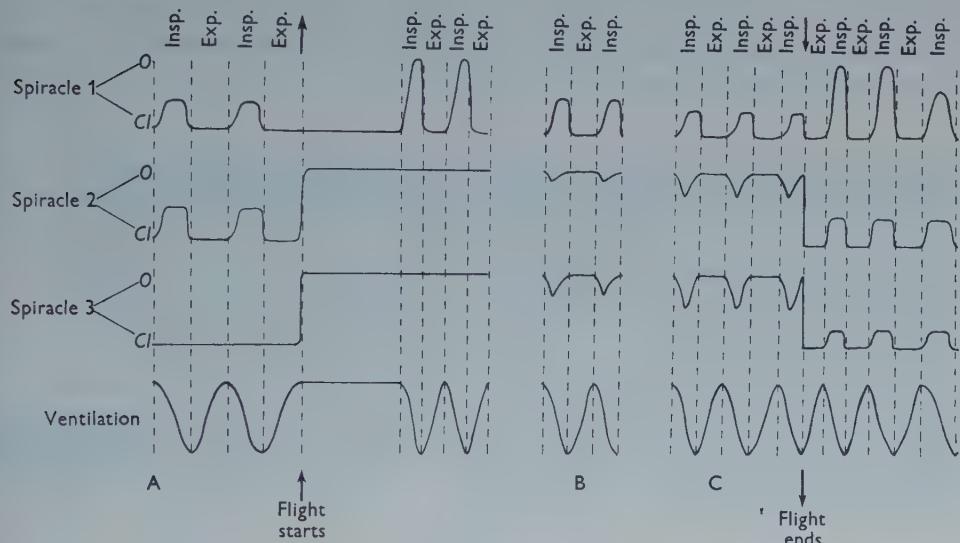


Fig. 1. Summary of the behaviour of the spiracles and of ventilation during flight. A, the start of flight. B, about 30 min. after the start of flight; incipient closing movements in spiracles 2 and 3. C, the end of flight. Cl, closed; O, open; insp., inspiration; exp., expiration.

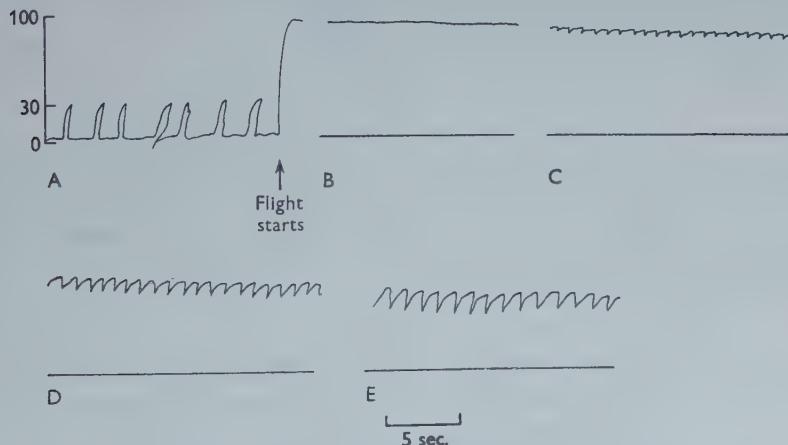


Fig. 2. Tracing from an actual mirror recording of spiracle 2 during flight. A, before flight. B, 5 sec. after the start of flight. C, 5 min. after the start of flight. D, 30 min. after the start of flight. E, 90 min. after the start of flight.

to the spiracle of a non-flying locust and so causing wide-opening; after a few seconds the muscle is able to close the spiracle completely against the mechanism.

Since the spiracle opens the instant flight starts and closes immediately it ceases, the reaction is unlikely to be mediated entirely by chemical stimulus. That it is not wholly a result of the action of carbon dioxide on the muscle membrane (Hoyle, 1960) has been shown by recording the impulses in the transverse nerve under oil during flight (Fig. 3). Initially the nerve is silent; later, as the incipient movements commence, bursts of impulses are recorded, but each burst comprises no more than about 6–10 impulses as compared with 50–80 at rest.

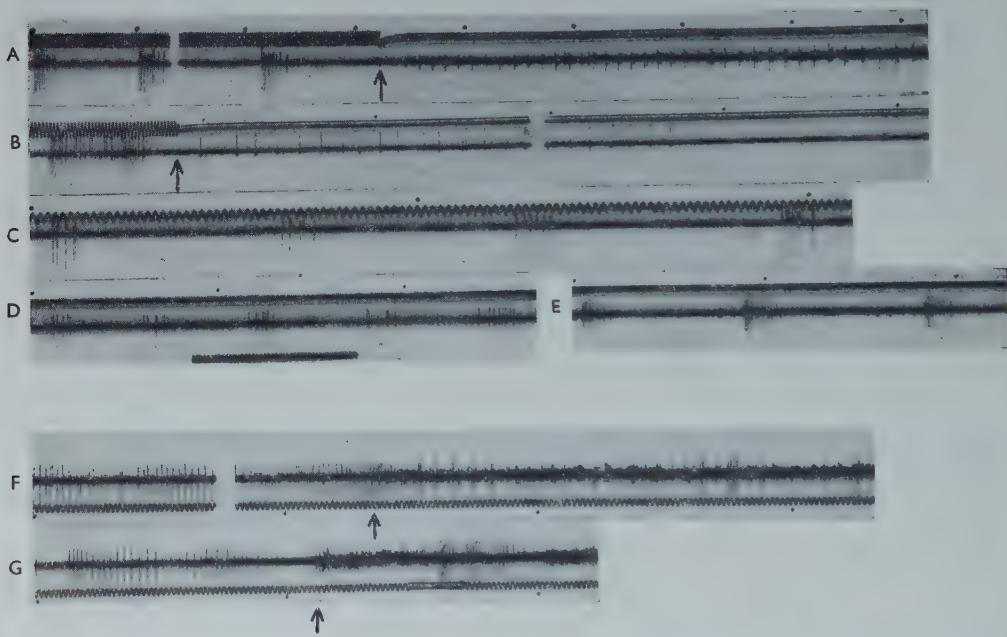


Fig. 3. Oscilloscope records from the transverse nerves of spiracles 2 (A–E) and 3 (F and G) during flight. A and B, flight starts at the arrow; bursts of motor impulses to the spiracle closer cease. C and D, records from the transverse nerve when the spiracles are making incipient closing movements. E, flight is very poor and the valve is nearly full closing. F and G, flight starts at the arrow but is weak. Opener impulses start at a high frequency, the spiracle opens wide, but irregular bursts of closer impulses continue. Time markers: 50 cyc./sec. (trace) and 1·0 sec. (dots).

Hoyle (1959) describes an inhibitory reflex causing opening of spiracle 2, which is initiated by strong contractions in the abdomen. Since the behaviour of spiracle 2 was unchanged when the locust was flown after the complete removal of the abdomen, this cannot be responsible for spiracle opening in flight. It would appear that a central inhibitory mechanism initiated by flight is responsible for the maintained opening of the spiracle.

Spiracle 3. The behaviour of this spiracle in flight is very similar to that of spiracle 2 (Fig. 1). Wide-opening is followed by incipient closing movements which start slightly earlier than those in spiracle 2. At the end of flight the spiracle closes fully, and subsequently opens with inspiration no more than 10–20 %. Oscilloscope recordings during flight (Fig. 3) show that wide-opening of the spiracle is accompanied by a high frequency (up to 200/sec.) of small (opener) impulses in the transverse nerve. When incipient closing starts, short bursts of larger impulses correspond to the weak contractions of the closer; as in spiracle 2 each burst may comprise no more than 6 impulses. They are superimposed on the opener impulses so that the closer operates against the opener—the latter acting like a rubber band.

This behaviour is again unlikely to be the result of chemical stimulation, and probably originates as a flight reflex in the central nervous system inhibiting closer and exciting opener impulses.

Spiracles 4–10. The behaviour of these spiracles is similar to that of spiracle 1. During flight they remain synchronized with abdominal ventilation.

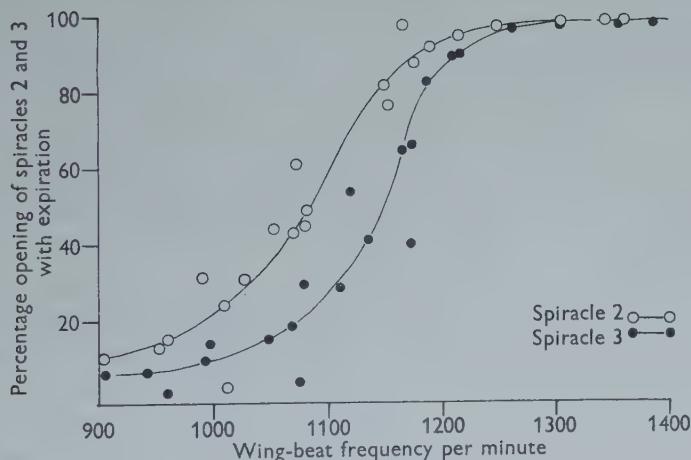


Fig. 4. The relation between the amount of incipient closing of spiracles 2 and 3 in flight (i.e. the percentage opening during expiration) and the wing-beat frequency.

The possible significance of incipient closing. Nearly simultaneous recordings were made of the wing-beat frequency and the amount of incipient closing of spiracles 2 and 3. Fig. 4, the results from several flights made by a male weighing 2.0 g., shows that there is a fairly close relation between the two, with spiracle 3 always slightly more closed than spiracle 2.

Wing-beat frequency is only one aspect of the total flight effort of the locust. If the incipient closing has any functional significance it would be expected to be more closely related to the metabolic rate.

From thoracic temperature measurements made in flight the metabolic rate has been calculated (Weis-Fogh, 1956b) and correlated with the amount of incipient

closing. Readings were commenced 10 min. after the start of flight and were made only when flight was steady and when no change in the wing-beat frequency had occurred for at least 2 min. The results (Fig. 5) show that the amount of incipient closing is more nearly related to the wing-beat frequency than to the metabolic rate. Weis-Fogh (1956a) mentions that there is a close relationship between lift and power output, but not between wing-beat frequency and power output. Since the size of the incipient closing movements appears to be more nearly related to the wing-beat frequency, it presumably has little functional significance.

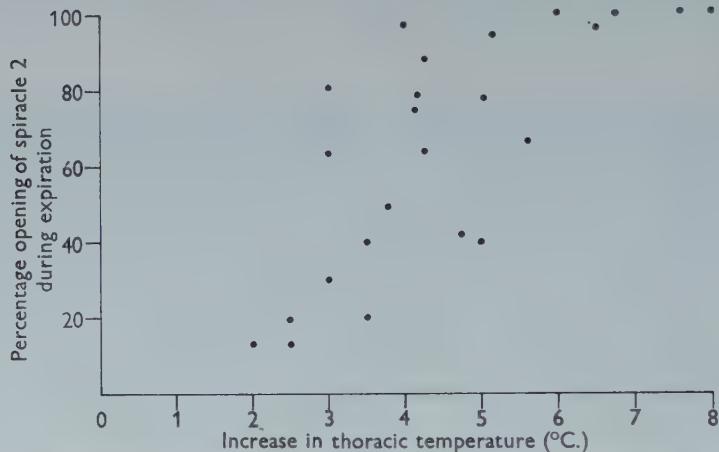


Fig. 5. The relation between the amount of incipient closing of spiracles 2 and 3 in flight and the excess temperature of the thorax over that of the air. (This is directly related to the metabolic rate—see text.)

This conclusion has been supported by the results of measuring the air flow through spiracle 2. The spiracle was cut out, waxed to the end of a long narrow glass tube, and air was driven through under a constant pressure of 3 cm. water. The time for the meniscus to pass between two fixed points was measured. The valves were set in various positions with wax and their maximum separation noted. This distance was then plotted against the air flow in ml./min. (Fig. 6). Point 1 on the graph indicates the separation of the valves due to muscle relaxation alone, and point 2 that due to the wide-opening mechanism. While the separation of the valves increases with wide-opening by 70%, the air flow increases by only 27%. In subsequent experiments in which the pressure was increased to 20 cm. water, wide-opening doubled the air flow. The pressure changes synchronous with wing movements do not exceed 1–3 cm., while those resulting from abdominal ventilation are as much as 20 cm. water (Weis-Fogh, 1960). Most of the incipient movements take place between 80 and 100% open where they can have hardly any effect on the air flow. On the other hand, very slight adjustment of the position of the valves, when they are almost closed, may be of great importance.

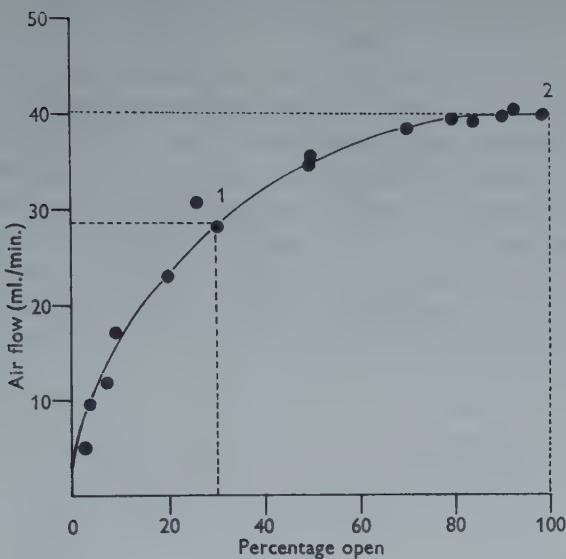


Fig. 6. The air flow through spiracle 2 at a pressure of 3 cm. water, with the valves set in different positions. Point 1, maximum opening at rest. Point 2, maximum opening in flight.

Modifications of the tracheal system and the continued synchronization of spiracle 1 during flight

The flight tracheal system. Weis-Fogh (1960) has pointed out the considerable isolation of the tracheae which supply the flight muscles from the remainder. This pterothoracic tracheal system comprises on each side (Fig. 7) the supra-ventral trunk running posteriorly from the ventral orifice of spiracle 1, with branches to the second and third legs, to the flight muscles and to the plexus of spiracle 2; a dorsally running trachea from the ventral orifice of spiracle 1, supplying the flight muscles and the main thoracic air-sacs; and finally tracheae from spiracles 2 and 3 to the flight muscles and the air-sacs.

The flight tracheal system joins the tracheal system of the remainder of the insect at the following points (Fig. 7).

(i) Branches from the anterior end of the main thoracic air-sacs which run into the dorsal cephalic tracheae. They are small; the ratio of their cross-sectional area to that of the cephalic tracheae is 1:14.

(ii) At spiracle 1 across the atrium from the ventral to the dorsal orifice. This route is only effective when the spiracle is closed, and then only when the opener is relaxed and the ventral orifice open (Miller, 1960*b*).

(iii) Across the ventral plexus of spiracle 1 into the leg trachea, and thence to the dorsal cephalic trachea via a small loop, the ratio of whose cross-sectional area to that of the dorsal cephalic trachea is 1:20.

(iv) From the plexus of spiracle 3 through a long thin air-sac to the longitudinal ventral trunk.

(v) From the plexus of spiracle 3 through branches which run posteriorly and anastomose with the alimentary tracheae.

Routes (iv) and (v) will be effective only when the spiracle is closed.

The trachea from the large anterior abdominal air-sac runs down close to spiracle 4, where it bends sharply to the posterior and then joins the longitudinal ventral trunk. At the bend the occluded remains of a branch into the tracheal plexus can be seen. In the more posterior spiracles the corresponding branch is normal. There is no connexion between the anterior abdominal air-sacs and the main thoracic air-sacs in spite of their contiguity. The former may have little respiratory significance and be more important in reducing the mechanical damping of the wing muscles (Weis-Fogh, 1953).

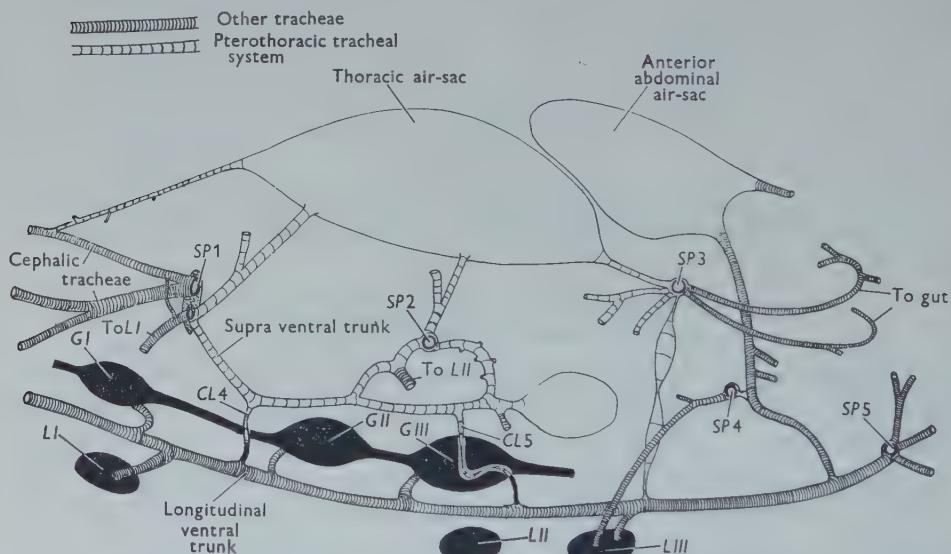


Fig. 7. Diagram of the pterothoracic tracheal system and its junctions with the other main tracheae. *GI*, *GII* and *GIII*, pro-, meso- and metathoracic ganglia; *LI*, *LII* and *LIII*, pro-, meso- and metathoracic legs; *SP*, spiracle; *CL* 4 and 5, degenerate cross-linking tracheae.

Two pairs of non-functional cross-linking tracheae run between the supraventral trunks and the longitudinal ventral trunks (Fig. 7). In the more anterior pair (*CL* 4), situated just in front of the mesothoracic ganglion, each trachea is flattened and, near its junction with the ventral trunk, filled with liquid. The ratio of its cross-sectional area to that of the ventral trunk is 1:47. The posterior pair (*CL* 5), level with the metathoracic ganglion, comprises longer tracheae entirely flattened and usually liquid-filled near the ventral trunk. The corresponding ratio is 1:25. In *Locusta migratoria* the equivalent cross-links are thinner and entirely liquid-filled.

In the first instar *Schistocerca* both pairs of cross-links are relatively larger (the corresponding ratios are for the anterior 1:2, and for the posterior 1:3) and appear

as normally functional tracheae. In the second instar they are flattened, and by the third they become liquid-filled.

A number of tests on the adult has demonstrated that these links never conduct air. Adults of all ages and both sexes have been inspected after long flights. The links have been observed during hyperventilation through windows glued to the sterna. Air has been blown into spiracles 1 and 2 under pressures of up to 20 cm. water. During some of these tests the flattened portions of the links were inflated, but the liquid was never expelled.

The large trachea from spiracle 4 supplies almost entirely the metathoracic leg. Since spiracles 2 and 3 supply principally the pterothoracic system, the main source of inspired air for the central nervous system and the rest of the locust is the dorsal orifice of spiracle 1.

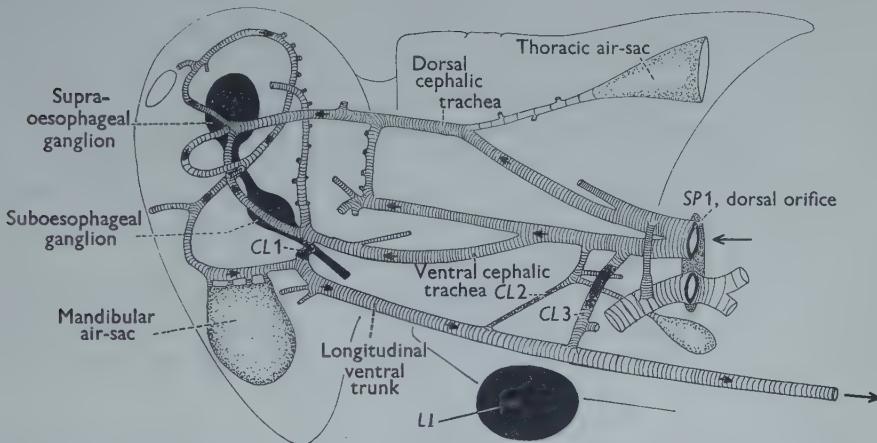


Fig. 8. Diagram of the main tracheae to the head and their relation to the dorsal orifice of spiracle 1.

The arrows indicate the probable direction of the movement of air resulting from abdominal ventilation. *LI*, prothoracic leg base; *CL* 1, 2 and 3, degenerate cross-linking tracheae; *SP* 1, spiracle 1.

Examination of the cephalic tracheal system, which is supplied by the dorsal orifice, has revealed three further pairs of degenerate cross-links (Fig. 8), the degeneration occurring in each case after the first instar. They all run between the ventral cephalic trachea and the longitudinal ventral trunk. The third (*CL* 3) is near the front legs and comprises on each side a broad flattened trachea, liquid-filled at its dorsal end. The second (*CL* 2) is a long, collapsed and partially liquid-filled air-sac close to the third, and the first (*CL* 1), inside the head, is a short trachea entirely liquid-filled. Tests, similar to those already described, suggest that none of these conducts air in the adult.

Their occlusion means that air can pass from the cephalic tracheae to the longitudinal trunks only through anastomosing air-sacs and tracheal loops close to the cephalic ganglia. Thus inspired air from the dorsal orifice of spiracle 1 goes straight to the brain and then down the ventral trunks to the thoracic ganglia. The continued

synchronization of spiracle 1 during flight will ensure the maintenance of this stream of air, thereby adequately ventilating the central nervous system.

Only after the first instar do regular synchronized movements of the spiracles commence, and a directed stream of air through the insect then becomes possible. Since all five pairs of cross-links become non-functional after the first instar, their occlusion is probably associated with this air-stream. Tracheae which have apparently outlived their usefulness are notorious for their persistence (Hamilton, 1931; Kramer, 1937; Smith, 1958). It is probable, therefore, that the occlusion of these cross-links enhances the air flow through the insect, directing it close to the cephalic and thoracic ganglia.

DISCUSSION

It has been shown that the opening of spiracle 2 with flight is controlled by a reflex mechanism. It was argued (Miller, 1960b) that the peripheral action of carbon dioxide on the muscle membrane is much more effective when the frequency of motor impulses is low; it seems most probable that the small number of motor impulses which reaches the muscle during incipient closing fails to cause more than a very weak contraction because of the presence of 5% carbon dioxide near the spiracle. The full closing immediately after flight is due to a greatly increased frequency of motor impulses. In this way the ganglion is effectively altering the sensitivity of the local reaction of the spiracle to carbon dioxide.

To summarize, spiracle 2 stays open for the first 5–10 min. of flight because it receives no motor impulses: subsequently it makes only very weak closing movements due to the combination of a small number of motor impulses and the peripheral action of carbon dioxide.

Locust flight muscle is non-fibrillar and the ratio of nerve impulses to muscle contractions is 1:1. This means that the size of the incipient closing movement is related to the frequency of flight motor impulses originating from the meso- and metathoracic ganglia. There would appear to be competition between an hypothetical flight centre, which inhibits impulses in the motor nerves to the spiracle closers, and a ventilation centre which rhythmically excites them. The higher the wing-beat frequency, the greater the frequency of impulses in the nerves to the flight muscles and the more complete the inhibition of the closer neurones. The rhythmical centre of ventilation is in the metathoracic ganglion, and a spiracle-inhibiting centre exists in the same ganglion, at least in the dragonfly (Miller, unpublished). This behaviour suggests the concept of negative induction (Pavlov, 1927), which may be described as 'a concentrated state of excitement which produces an inhibition around itself' (Konorski, 1948).

During the flight of the locust the 'concentrated state of excitement' of the flight motor neurones may be adequate to induce a field of inhibition around themselves, and possibly internuncial fibres, which supply the closer motor neurones of spiracles 2 and 3, have synapses within this field. More simply, the flight centre may supply inhibitory nerves to these synapses: such a scheme is represented in Fig. 9.

Several possible advantages might arise from keeping the pterothoracic tracheal system isolated. Volume changes induced by flight movements might be able to ventilate the pterothorax more efficiently through the open spiracles 2 and 3, since the system forms an isolated unit. High carbon-dioxide tension and oxygen lack might to some extent be limited to the pterothorax, while the abdomen continues to ventilate the central nervous system through spiracle 1, and does not

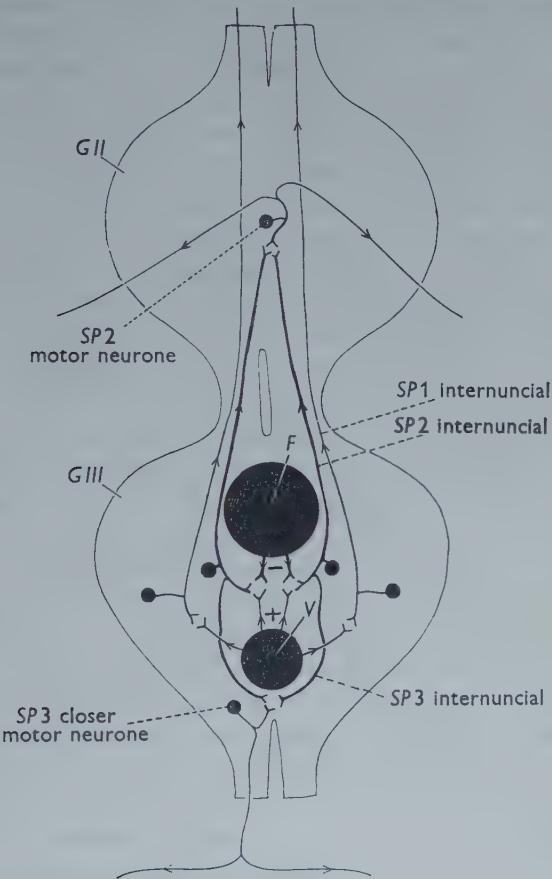


Fig. 9. Scheme to account for the inhibition by negative induction of the closer motor neurones of spiracles 2 and 3 in flight. *F*, flight centre; *V*, ventilation centre; —, inhibitory nerves; +, excitatory nerves; other abbreviations as in Fig. 7.

draw 'used air' into the longitudinal trunks. The ability of carbon dioxide to diffuse through animal tissues faster than oxygen (Krogh, 1919) probably means that pterothoracic isolation is more important in preventing the withdrawal of oxygen from other tissues (in particular the central nervous system), than in limiting high concentrations of carbon dioxide to the pterothorax.

That oxygen lack, and possibly carbon dioxide accumulation, are partially limited to the pterothorax is suggested by a number of observations. Auxiliary ventilating mechanisms seldom function in flight; nevertheless, when a resting locust

is placed in an atmosphere of 5% carbon dioxide, they continue for at least an hour without diminution. 5% carbon dioxide and 15% oxygen are concentrations commonly occurring in the main thoracic air-sacs in flight (Weis-Fogh, 1960), so that either the ventilatory centres are depressed by flight or they remain unstimulated. The results of injecting 5% carbon dioxide into the mandibular air-sac of a flying locust, giving rise to considerable hyperventilation, show that the ventilatory regulation centres are not depressed but remain unstimulated. The considerable increase in ventilation and the wider opening of spiracle 1, which occur momentarily after flight, may be explained by the closing of spiracles 2 and 3 and the flooding of the ganglionic regulatory centres with pterothoracic gases which are driven anteriorly by abdominal expiration.

At the beginning of flight, and subsequently if there is a sudden increase in wing-beat frequency, spiracle 1 opens fully with inspiration as a result of a strong and maintained contraction of the opener. When the spiracle is closed, this contraction constricts the ventral orifice completely, but when the closer relaxes the orifice opens a limited amount (Miller, 1960b). The constriction of the ventral orifice prevents gases in the pterothorax from being blown by abdominal expiration across the atrium, into the cephalic tracheae and so to the central nervous system. Opener contractions are initiated by carbon dioxide in the head and gases will still be able to reach the head from the thoracic air-sacs through the cephalic tracheae (Fig. 8). However, the sudden initial contraction of the opener at the start of flight suggests it may then be controlled by a nervous reflex, independent of chemical stimulation.

Hoyle (1959) has drawn attention to the rich tracheation of the closer muscle of spiracle 2. The small tracheae to the muscle arise from an air-sac which is itself in direct communication with flight muscle air-sacs. Moreover, a 'through trachea', arising from this air-sac, passes across the muscle sending small twigs into it, and then joins a larger trachea which leads straight into the spiracle plexus. This may enable the muscle to sample gases on their way to the exterior during flight, and seems to refute the suggestion that to have a carbon-dioxide sensing mechanism in the spiracle is to have it 'in the worst possible situation, that is, almost outside the insect' (Case, 1957). 'Through tracheae' have been observed in association with the dragonfly spiracles, which appear to be controlled by a comparable local reaction to carbon dioxide (Miller, unpublished).

SUMMARY

1. During normal flight of the desert locust, auxiliary ventilating mechanisms do not appear, and dorso-ventral abdominal pumping continues at increased frequency and amplitude. When flight stops hyperventilation together with auxiliary forms appear briefly. Removal of the abdomen has shown that pterothoracic and neck ventilation are adequate for sustained flight.

2. Spiracles 2 and 3 open wide during flight: when flight is weaker they make incipient closing movements. A central inhibitory reflex controls their activity,

in addition to the peripheral action of carbon dioxide on spiracle 2. The incipient closing movements are shown not to have a functional significance; they are probably the expression of two competing mechanisms, and may arise by negative induction.

3. Spiracles 1 and 4-10 remain synchronized with ventilation, and thereby permit adequate ventilation of the central nervous system.

4. The isolation of the pterothoracic tracheal system is enhanced by the occlusion of two pairs of cross-links. The occlusion of a further three pairs in the prothorax and head ensures that the head has priority on the inspired air.

5. The occlusion of all the cross-links takes place after the first instar, at which time spiracle synchronization first regularly appears and a directed airstream becomes possible.

6. In flight there are two largely independent ventilating systems. The first, a two-way system, ventilates the flight muscles through the open spiracles 2 and 3 and is pumped by the flight movements. The second, a one-way system, ventilates primarily the central nervous system and is pumped by the abdomen, in through the dorsal orifice of spiracle 1, and out through spiracles 5-10.

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NEURAL MECHANISM OF HEARING IN INSECTS

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INTRODUCTION

Since the work of Wever & Bray (1933) many electrophysiological studies have been made on sound reception in insects and it has been established that their receptive organs can readily detect the direction of sound and can discriminate its intensity, but not its frequency (Pumphrey, 1940; Autrum, 1955). More recently Haskell (1956, 1957), Haskell & Belton (1956), and Roeder & Treat (1957) have studied this problem further, and the ultrasonic reception in noctuid moths has also been confirmed by electrophysiological methods.

From the view point of the comparative auditory physiology the present authors have tried to clarify the neural mechanism of hearing in insects by means of the same technique as they have used in recent studies of mammals. This report will be concerned with the results obtained from several kinds of insect which are very common in Japan.

MATERIALS AND METHODS

As material, insects belonging to four families: Cicadidae (*Tanna japonensis*, *Platypleura kaempferi* and *Graptopsaltria nigrofuscata*); Acridiidae (*Oxya japonica* and *Locusta migratoria danica*); Tettigoniidae (*Mecopoda elongata*, *Gampsocleis buergeri* and *Hexacentrus japonicus japonicus*); and Gryllidae (*Xenogryllus marmoratus* and *Homoeogryllus japonicus*) were used. Experiments were performed during summer and autumn when those insects were obtainable; the experiments were made on Cicadidae early in summer, then on Tettigoniidae and Gryllidae, and late in autumn on Acridiidae.

These families have their tympanal organs in different parts of their bodies; Tettigoniidae and Gryllidae have them at the proximal end of the tibiae of the first forelegs, and Acridiidae and Cicadidae at the abdomen. In order to expose the tympanal nerve the body of the animal was fixed with pins on a cork board upside down and the exoskeleton was removed from the region of the thoracic ganglion with which the tympanal nerve connects, that is, from the prothoracic ganglion in Tettigoniidae and Gryllidae, the mesothoracic ganglion in Cicadidae and the metathoracic ganglion in Acridiidae. In studies on the cercal nerve which also shows response to sounds the animals were pinned in the normal position and the sternites were cut away from the posterior half of abdominal segments at the dorsal side; the intestine, rectum and trachea were removed to expose the last abdominal ganglion.

After the tympanal nerve or the cercal nerve had been exposed, the nerve was cut as close as possible to the ganglion and then the cut end was raised into the air on a silver wire electrode of $200\ \mu$ in diameter, by which the responses of the nerve to sound stimuli were recorded.

In order to obtain the response of a single neuron to the sound stimuli from the tympanal nerve bundle, 3 M-KCl capillary micro-electrodes with ohmic resistances between 30 and 50 M Ω were used. The electron-microscope revealed the tip diameters to be less than $0.2\ \mu$. The electrodes were slowly introduced into the root of the tympanal nerve by a micromanipulator through the small hole which was made at the ventral surface of the thoracic ganglion. The cathode-follower pre-amplifier with a very low grid current (10^{-12} A.) was assembled with Z-729 by triode connexion. As main amplifiers an r.c. and, if necessary, high-gain d.c. amplifier were employed simultaneously. An indifferent electrode was placed on an exposed abdomen.

Recordings were made photographically with a cathode-ray oscilloscope, the beam of which was divided into three channels by means of electronic switches. In this way responses of neurons, time signals and sound waves could be displayed simultaneously. Most records were obtained on running film by the use of a long-recording camera, the stimulus sounds being presented in succession.

Four loud-speakers, one of which was for ultrasonic waves, were used as sound sources and delivered automatically in succession through an attenuator 44 tone bursts of different frequencies fixed between 30 and 100,000 c./s. The frequency characteristics of those four combined speakers was examined and found to be flat within ± 5 db. The duration of tone bursts was of the order of scores milliseconds, were varied in will, if necessary. The sound stimuli were delivered to the auditory organ of the animal in a free field. During the presentation of sounds their intensity was controlled by the attenuator.

Our reference level (zero db.) corresponded to a sound intensity of approximately 100 db. above the lowest average human threshold at 2000–3000 cycles.

Operated animals were isolated in a sound-proofed room and directed towards the loud-speakers at a distance of about 50 cm. The temperature in the room was kept by air conditioning at about 27° C.

RESULTS

(i) Frequency range of response

The frequency range in which the tympanal or cercal nerve was activated by tone bursts of a certain intensity could be determined on the records which had been photographed on a running film. Such serial records obtained by tone bursts in successive different intensities gave the thresholds of the nerve responses for respective frequencies of sounds. Thus the response ranges of these nerves, in other words those of the end-organs innervated by them could be represented by plotting the thresholds, the frequency of sound being shown on the abscissa and the intensity in decibel units on the ordinate (Fig. 1).

Fig. 1 shows the average response ranges which are obtained from Cicadidae, Acrididae, Tettigoniidae and Gryllidae.

(A) In Cicadidae the responses to sound stimuli were obtained from the tympanal nerve bundle which connects with the mesothoracic ganglion. Fig. 1 A shows the response ranges of the tympanal nerves of three species of Cicadidae. Those response ranges are the average threshold curves of the tympanal nerves of male specimens in each species. The sexual difference in the response range was examined in *Tanna japonensis* and *Platycleura kaempferi*, and no particular difference was found except for the slightly higher threshold in the female. The response ranges

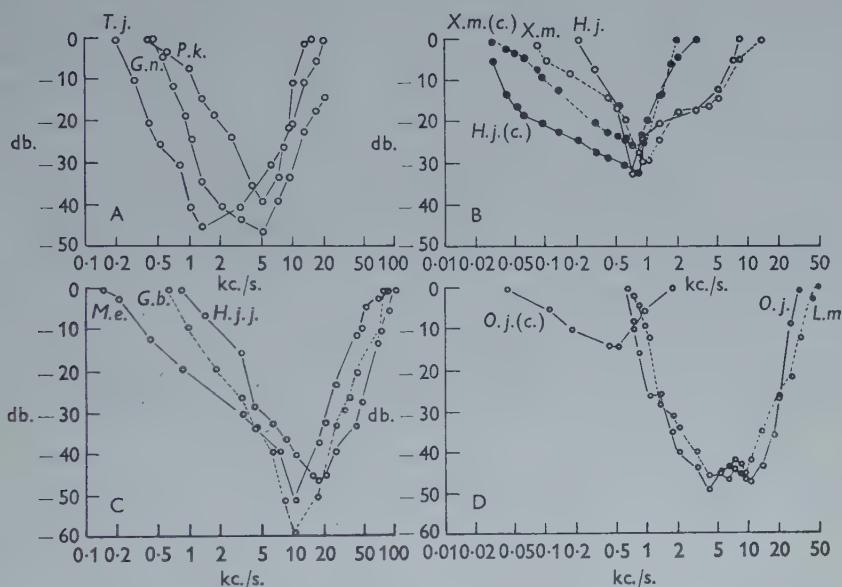


Fig. 1. Auditory response ranges of both the tympanal organs and the cercal hair sensillae of ten species belonging to Cicadidae (A), Gryllidae (B), Tettigoniidae (C) and Acrididae (D). The ordinate and the abscissa represent the intensity of sound in decibel unit and the frequency in kilocycle, respectively. The response range of tympanal organ of each species is marked with its initial, and also that of cercal hair sensilla with c. in brackets together. It is noted that the tympanal organs of Tettigoniidae and Acrididae respond to unusual ultrasonic stimuli.

in which the tympanal nerves were activated by the sounds of 0 db. for respective species were as follows: in *Tanna japonensis*, from 0.2 to 20 kc./s.; in *Platycleura kaempferi*, from 0.4 to 15 kc./s.; and in *Graptopsaltria nigrofuscata*, from 0.45 to beyond 20 kc./s. The most effective frequencies were 13, 5 and 5 kc./s., respectively.

(B) The insects of the family Gryllidae are commonly provided with well-developed cerci. Fig. 1 B shows the average response ranges of the tympanal and the cercal nerves of *Homoeogryllus japonicus* and *Xenogryllus marmoratus*.

The most effective frequencies for the tympanum and for the cercal hair sensilla were almost the same (*H.j.* and *H.j. (c.)*, *X.m.* and *X.m. (c.)*). However, the response ranges to sound stimuli of the tympanal organ and the cercal hair sensilla in *Homoeogryllus japonicus* and also *Xenogryllus marmoratus* were from 0.2 to 8 kc./s. (*H.j.*),

from 0.03 to 3 kc./s. (*H.j. (c.)*), from 0.08 to 13 kc./s. (*X.m.*) and from 0.03 to 2 kc./s. (*X.m. (c.)*), respectively. In *Homoeogryllus japonicus* it was recognized that the cercal nerve can respond to a tone burst of up to 300 c./s. with synchronous discharge and can respond to gross air movements with bursts of spikes. Similar responses were also recognized in Haskell's studies (1956) with certain species of Acridiidae.

(C) Fig. 1C shows the average response ranges obtained from the tympanal nerves of three species of Tettigoniidae. In these insects the tympanal organ is situated as described above at the tibia of the first foreleg and the tympanal nerve is involved in the nerve going out to the first foreleg from the prothoracic ganglion.

The response ranges of each species were as follows: in *Gampsocleis buergeri*, from 0.6 to 75 kc./s.; in *Mecopoda elongata*, from 0.14 to 85 kc./s.; and in *Hexacentrus japonicus japonicus*, from 0.8 to 100 kc./s.; the most effective frequency for each species being 10, 10 and 17 kc./s., respectively.

(D) The responses were obtained for two species of Acridiidae from the proximal part of the tympanal nerve which connected with a metathoracic ganglion, or from its distal part which connected with the chordotonal organ attached to the tympanal membrane. No remarkable differences were found between the two records apart from the tendency to flatness on the response curve in the region of the most effective frequency in the former case.

In Fig. 1D the curve *L.m.* represents the response range of the tympanal organ of *Locusta migratoria danica*. The response range is from 0.6 to 45 kc./s., the most effective frequency range being 4–9 kc./s. The response range and the most effective frequency of the tympanal organ of *Oxya japonica* are from 0.6 to 30 kc./s. and from 4 to 10 kc./s., respectively, while those two of the cercal hair sensilla were from 0.04 to 1.7 kc./s. and from 0.4 to 0.5 kc./s. (curve *O.j. (c.)*), respectively.

It is of great interest that the tympanal organs of Acridiidae and Tettigoniidae can respond to unusually high-frequency ultrasonic waves and that the most effective frequency of the latter is very high, even higher than 10 kc./s. The response ranges of the other two families, Cicadidae and Gryllidae, are found to be almost within that of man. As seen in Gryllidae and Acridiidae, the tympanal organ takes charge of the high-frequency sound, while the cercal hair sensilla take charge of the low-frequency sound. These insects thus can receive sounds over a wide frequency range with their two separate organs.

(2) Relation between sound production and sound reception

The responses of the tympanal nerve to natural stridulations of groups of insects were recorded from the tympanal nerves of *Meimuna opalifera* (♀), *Gampsocleis buergeri* (♂), and *Mecopoda elongata* (♂) simultaneously with the stridulatory sound. Fig. 2 is a case of *Gampsocleis buergeri*. In each figure the upper, middle and lower beams represent the nerve response, the sound of natural stridulation, and the time mark of 10 msec., respectively. When the tympanal organ is stimulated by the natural stridulation of a group the tympanal nerve sends the volleys of impulses which are synchronous with the pulsatory sound. It has been noted since the

original work of Pumphrey (1940) that the tympanal organ receives effectively the pulsatory sound in cicadas (Pringle, 1953, 1954), in moths (Roeder & Treat, 1957) and in the grasshopper (Haskell, 1956, 1957).

It may now be asked what component frequencies were involved in the stridulatory sound. The stridulatory sound recorded with a tape recorder which was specially designed for both sonic and ultrasonic waves at the Technical Laboratory of Japan Broadcasting Corporation, was analyzed by means of the Sona-Graph. The stridulatory sound is mere 'noise' except for that of *Homoeogryllus japonicus*, so that the results of sound analysis appear as a continuous sound spectrum. The case of *Gampsocleis buergeri* is shown in Fig. 3. In the figure, C shows the wave-form of the stridulatory sound which was recorded with a precision microphone. B is the sonogram, in which the intensities of component sounds involved in the stridulatory sound are shown as the grade of darkness. In the sonogram the temporal

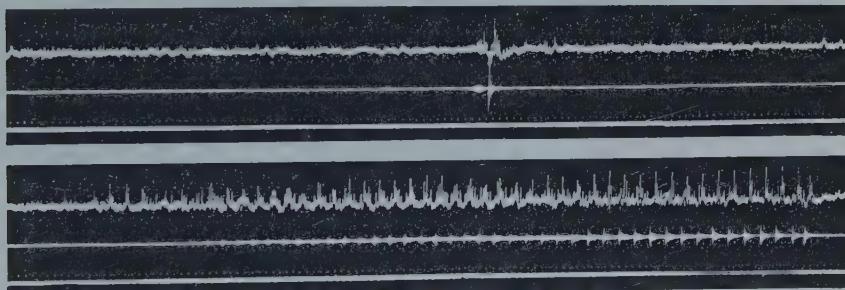


Fig. 2. Response of the tympanal nerve of *Gampsocleis buergeri* to the natural stridulatory sound of the company. The top, the middle and the bottom beam represent the nerve response, the wave of the stridulatory sound and the time scale of 10 msec., respectively.

change of both the frequency and the intensity of sound are well shown. A is the sectioner, which represents with the height of bar the intensities of component sounds involved at the moment indicated by an arrow. The stridulatory sound of *G. buergeri* contained a high-frequency sound of 40 kc./s., and the sounds of about 8 and 12 kc./s. were especially dominant in it. Those frequencies are called in this article the upper frequency limit and the dominant frequency range, respectively. It was also found by sonograms and sectioners that the stridulatory sound of *Mecopoda elongata* had an upper frequency limit of 40 kc./s. and a dominant frequency range of 10 kc./s., and that of *Homoeogryllus japonicus* an upper frequency limit of 20 kc./s. and a dominant frequencies of 0.7 and 7 kc./s.

On the other hand, the most effective frequencies in the tympanal organs of these insects were 10, 10, and 0.7 kc./s., respectively (C, C and B of Fig. 1). It is a matter of surprise that the dominant frequency range involved in the sound produced by the animal itself shows good agreement with the most effective frequency range in the tympanal organ of that animal. Though the author did not actually analyze the stridulatory sound of Acrididae, Haskell's studies (1957) show that the dominant frequency of the wing-beat sound of desert locust is about 4 kc./s.

The most effective frequency of the tympanal organ of Acridiidae obtained by the present authors is about 4-9 kc./s.: *Locusta migratoria danica* 4-9 kc./s., and *Oxya japonica* 4-10 kc./s. (D of Fig. 1). Such a good agreement between sound production and its reception must show that stridulation has played an important role in communication among insects. The sound reception of Tettigoniidae and Gryllidae was studied only on the male because of the difficulty in getting females. It is

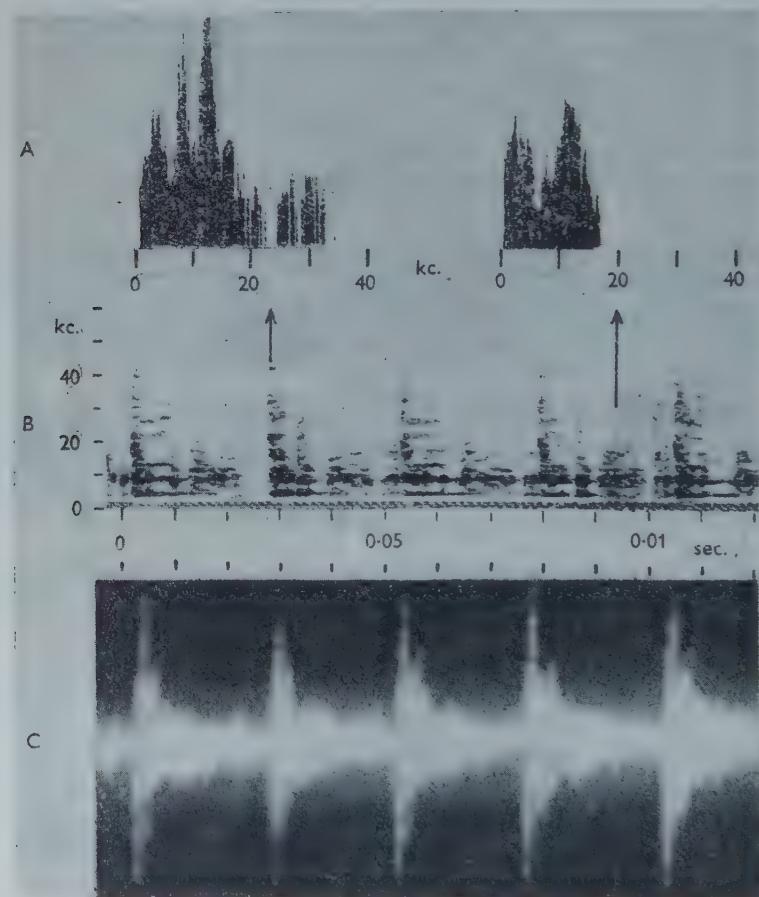


Fig. 3. Stridulatory sound of *Gampsocleis buergeri* (C) and its analysis (A and B). See text.

inconceivable that in those insects there are sexual differences in sound reception between male and female. Indeed, the present authors confirmed in other species, for instance, Cicadidae and Acridiidae, that there were no sexual differences in sound reception.

Haskell's study (1956) also reported the same results in Acridiidae. This too may be quite reasonable from the view point of mutual communication. However, in the case of Cicadidae there was disagreement between our results obtained from

the tympanal nerve of these insects and the sound analyses made by other authors. Quite recently Hagiwara of our laboratory analysed the natural sounds of several kinds of Cicadidae by the same methods as we used and the results were also considerably different from the analyses of sound reception. The reason for these discrepancies may be the variable tension of the tympanal membrane resulting from the creasing action of the detensor muscle of the cicada (Pringle, 1953).

(3) Sound localization

It is conceivable that the ultrasonic wave produced by stridulation may play a critical role in the orientation of the insect. The production as well as the reception of the ultrasonic waves was confirmed electrophysiologically on bats (Griffin & Galambos, 1941) and noctuid moths (Roeder & Treat, 1957). The present examination was performed on the tympanal organ of *Locusta migratoria danica* to find the difference of sensitivity of the tympanal organ to sonic as well as to ultrasonic waves coming from various directions. The tympanum makes an angle of about 45° backward with the body axis and is partly protected by a shield of exoskeleton. The material was mounted with a manipulator and a cathode-follower pre-amplifier on a rotatable round table and about 1.25 m. distant from the loud-speakers which were placed at the same height. The thresholds of responses of the tympanal nerve were measured for sonic and ultrasonic waves coming from different directions as shown in Fig. 4. Concentric circles represent the intensity of sound, 0, -10, -20, -30, -40 and -50 db., respectively. Eccentric curves represent the thresholds of nerve responses to the incident waves from different directions of 6, 10 and 30 kc./s., respectively. The solid lines show the threshold of the tympanal organ which faced the loud-speakers and the dotted lines that of the organ on the opposite side. The spaces between the solid and dotted lines show the differences of sensitivity between right and left tympanal organs. The higher the frequency, the more distinct was the difference of threshold. The threshold was highest for the incident wave coming from the direction of the head and lowest for the wave coming perpendicularly to the body axis. The tympanal organ has sharp directional sensitivity to sounds, especially to ultrasonic waves, in spite of its small size.

(4) Frequency discrimination

Pumphrey (1940) and later Autrum (1955) suggested that insects might be unable to discriminate the frequency of sounds. No definite evidence for it has, nevertheless, been found. It thus seemed likely that the recordings of responses of single auditory neurons to sonic and ultrasonic stimuli might provide a conclusive answer. Attempts were therefore made to record the response of a single neuron from the tympanal nerve of a tettigoniid by the use of a superfine microelectrode.

As already described the tympanal nerves of these animals connect with the prothoracic ganglion. The ganglion and also the tympanal nerve are covered with a hard sheath, through which the insertion of a micro-electrode was found to be almost impossible. An electrode was therefore inserted into the nerve bundle through a small hole which was made at the ventral surface of the prothoracic

ganglion by cutting the sheath. The recordings of the responses of single units were generally successful in the very superficial layer but not in the deep layer. By histological studies it was found that in the ganglia of the nerve cord the nerve cells were in general grouped most densely at the ventral side. Thus it is highly probable that many of the responses obtained might have come from the nerve cells

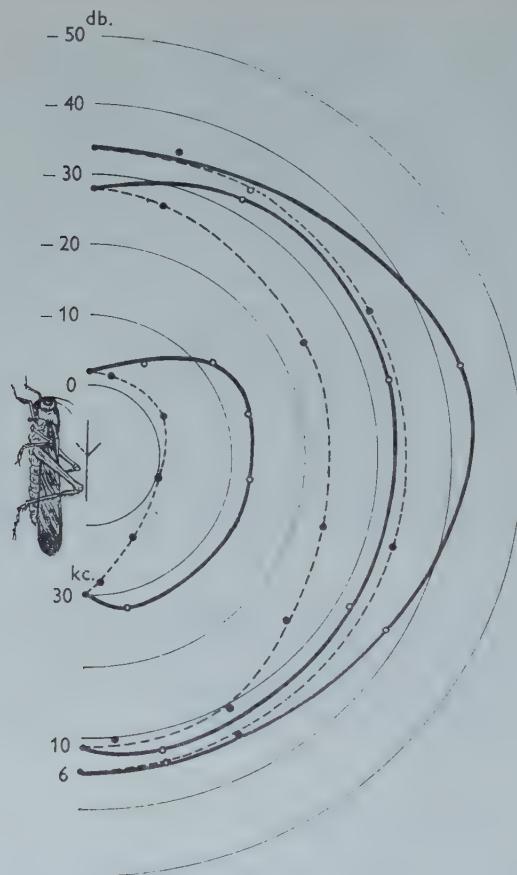


Fig. 4. Directional sensitivity of the tympanal organ of *Locusta migratoria danica*. The semi-circles represent the intensity of the sound of 0, -10, -20, -30, -40 and -50 db. from the inside, respectively, solid and dotted curves represent the thresholds of left (dotted) and right (solid) tympanal organs for the sound of 6, 10, 30 kc./s. coming from various directions. The position of the insect and the directions of tympanal membranes against the body axis are shown.

The recordings were mostly made extracellularly and very rarely intracellularly, and even when they were made from the ganglion itself the responses recorded from the superficial layers were clearly distinguished in their pattern from those of large nerve fibres involved in the connectives.

The response ranges of single neurons were determined by the same method as described above. Those ranges obtained from more than ten neurons were referable

to two types. One type had a response range of 3–60 kc./s. and the most effective frequency at about 10 kc./s. (Fig. 5), while the other had a response range of 0·6–30 kc./s. and the most effective frequency at 6–7 kc./s. The neuron activated by sounds of higher frequencies was relatively easily obtained, while the neuron activated by those of lower frequencies was rather difficult to find. The latter was obtained only when the isolation of the unit was incomplete. This fact suggests that the neurons responding to the sounds of lower frequencies are of small size,

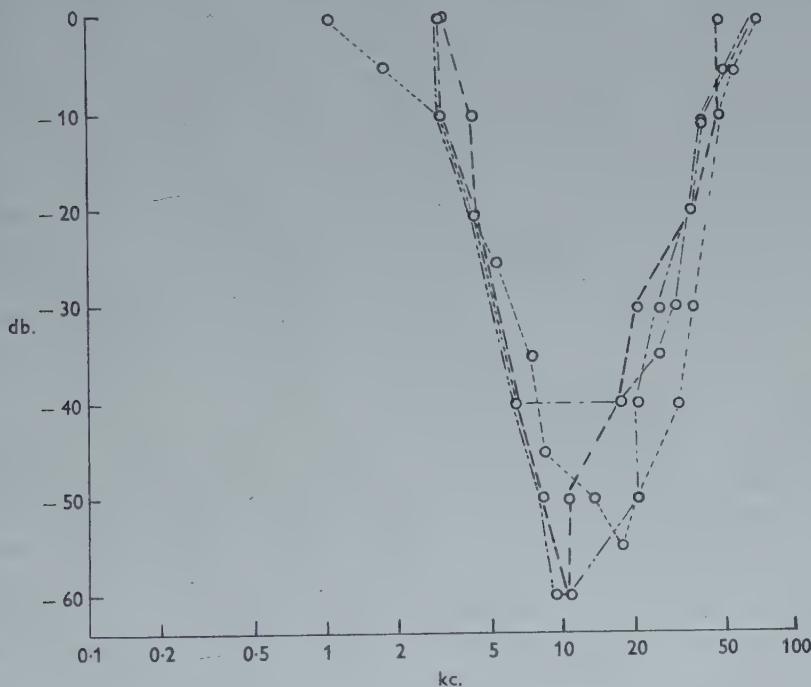


Fig. 5. Response ranges of 4 tympanal single neurons recorded with a superfine micro-electrode (*Gampsocleis buergeri*).

namely, thin fibres and small cell bodies. The neurons activated only by sounds of higher frequencies were more sensitive to stimuli than the others activated by sounds of lower frequencies. The tympanal nerve of noctuid moths is said to consist of two fibres (Eggers, 1919), which can be distinguished only by the difference in threshold of their respective end-organs for stimulating sounds (Roeder & Treat, 1957). More details of the nature of these two fibres in moths are needed. In relation to these results, it is of great interest that a large number of fibres which compose the tympanal nerve of *Gampsocleis buergeri* can also be referred to only two types.

On the neuron which responded to the higher frequency range of sound the relation between the number of spikes per second and the intensity of stimulus in decibels was explored for different stimulus frequencies. The curves were sigmoid

and almost parallel to one another for sounds with different frequencies. It is quite obvious that a neuron responds with the most frequent spikes to that sound to which the neuron is the most sensitive, among sounds with different frequencies but of the constant intensity, and it is also quite certain that there are various sounds with different frequencies and intensities which produce the same responses from the same neuron. Therefore a single auditory neuron cannot discriminate the frequency of sound, but can discriminate the change of its intensity.

DISCUSSION

Based upon the experimental results described above, the problems of frequency discrimination and recognition of the group in insects will be discussed. Frequency discrimination cannot of course be performed by a single auditory neuron as described above. If the insects have the ability to discriminate the frequency of sound, they should have many nerve fibres, with different response ranges. However, only two types of nerve fibres having different response ranges have been found in the tympanal nerve of *Gampsocleis buergeri*. Therefore it is concluded that the tympanal organ of insects has almost no ability to discriminate frequencies. However, it cannot be concluded from this fact alone that insects have no ability at all to discriminate the frequencies of sounds, because they have in addition many hair sensilla on various parts of the body. It can be shown as a notable example that there is the distinct difference in the response range between the tympanal organ and the cercal hair sensilla in *Homoeogryllus japonicus* and in *Oxya japonica* (Fig. 1). The tympanal organ which is exclusively adapted to sound stimuli responds to relatively high-frequency sounds, while the hair sensilla responds to relatively low-frequency sounds. It is indeed true that each of these receptors itself is almost unable to discriminate the frequency of sound. But generally speaking, it may not be so, because records obtained from the connective between the brain and the suboesophageal ganglion, which will be reported elsewhere, show that the impulses in response to sound stimuli are transmitted to the brain from the various parts of the body through different fibres which show different response ranges. Insects must discriminate stimulus frequency from the temporal and spatial pattern of impulses of many fibres which are sent to the upper brain, but of course such discrimination may not be sharp.

From the present experimental results alone the authors cannot discuss what role stridulation plays in mutual communication, but it may be said at least that stridulation does play some important role in natural communication from the fact that the most effective frequency range of the tympanal organ shows good agreement with the most dominant frequency range involved in the stridulatory sound produced by the insect itself. Such agreement also means that the stridulatory sound of the insect can most effectively stimulate the tympanal organ of the group. The stridulatory sound consists of pulsatory component sounds. If the tympanal organ of the insect has no ability at all to discriminate frequencies, the insect may still recognize its group solely by the rhythm of the pulsatory sound. Moreover,

it has been found that the insect may discriminate the complex sound by impulses coming not only from the tympanal organ, but also from various sound receptive organs which are widely distributed on the whole body. Thus the insects may recognize their group by discriminating sounds by means of the temporal and also spatial patterns of impulses which are sent up to the upper brain through many different nerve fibres in the cord. The functional analysis of the central nerve cord element is needed.

It is well known that the ultrasonic wave is very useful for the detection of the direction of sound. In this sense the fact that some insects belonging to Acridiidae and Tettigoniidae can produce ultrasonic waves and hear them is a matter of interest. It is indeed surprising that many insects have the ability to produce and to respond to ultrasonic waves. Such a function in insects must also be very important from the ecological point of view. The present studies have confirmed that the stridulatory sounds produced by certain insects involve high-frequency and even ultrasonic waves and the most effective frequency of sound to the insect ear is found to be the dominant frequency involved in the stridulatory sound. For natural communication and also for localization of sound, the higher the dominant frequency the more useful the sound should be to the insect. Insects of various species seem to live in a world of different sounds from ours.

SUMMARY

1. The frequency response ranges of the tympanal and cercal nerve were measured in ten species belonging to four families, Cicadidae, Acridiidae, Tettigoniidae and Gryllidae. The tympanal organs of Acridiidae and Tettigoniidae responded to ultrasonic waves and the most effective frequency was very high (> 10 kc./s.), while the response ranges of the other two families, Cicadidae and Gryllidae, were within that of man. The response ranges of the cercal nerves (lower) and tympanal nerves (higher) were partly overlapping.
2. Stridulation consisted of pulsatory sounds and had species-specific rhythms, to which the tympanal nerves responded with synchronous discharge.
3. The dominant frequency range involved in stridulation agreed well with the frequency range to which the tympanal organ of the same insect was most sensitive.
4. The threshold of the tympanal nerve varied with different directions of incident sound, especially for ultrasonic waves, indicating the possibility of directional sense.
5. Tympanal neurons of *Gampsocleis buergeri* were referable to two types having different response ranges.
6. The curves relating number of spikes per second to intensity of stimulus were sigmoid and almost parallel for different frequencies.
7. In Discussion it is pointed out that although no single receptor organ is able to discriminate stimulus frequency, an insect which has different sound receptors on various parts of its body may have some power of discrimination.

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NEURONAL PATHWAYS AND SYNAPTIC CONNEXIONS IN THE ABDOMINAL CORD OF THE CRAYFISH*

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As material for the study of functional connexions within a central nervous system, invertebrates with ganglionated nerve cords appear to offer advantages absent elsewhere. In such animals it should be possible to lead off from the axons which transmit the information from one synaptic region to another at all levels in the connectives, provided there are no cells or synapses in these connectives. In the crayfish, it has been previously shown that such an analysis is indeed possible, at the level between the 'brain' and the remainder of the ventral cord. It was found (Wiersma, Ripley & Christensen, 1955) that many interneurones respond to stimulation of several segments of the body, usually to stimulation of homologous areas of the individual segments. Three fundamentally different ways in which the inflow to different ganglia may become integrated in this way were suggested by Wiersma (1958), and are illustrated diagrammatically in Fig. 1. To gain further information about the occurrence of these different types, it was desirable to obtain recordings from interneurones at a lower level in the cord where the actual integration takes place. The isolated abdomen preparation proved highly suitable for this purpose. The number of fibres in each connective here is still smaller than that in each circumoesophageal commissure (about 1200 as against about 2000), and it was found possible to obtain from the cord preparations in which unit responses were clearly recognizable with not much more difficulty than from the commissure. Altogether it was possible to account for some 75 definite entities as against 100 in the commissure. In both places some of these are not single fibres but bundles of primary sensory fibres with similar properties. In the cord such bundles are more frequent than in the commissure. The nature and distribution of these entities will be described elsewhere; in the present paper their physiological properties will be discussed with special reference to the evidence they provide for the existence of these three possible types of integration. The results will show that the one in which a single interneurone collects impulses from different ganglia (scheme C of Fig. 1) is certainly realized in many of these interneurones.

MATERIALS AND METHODS

Both sexes of the crayfish *Procambarus clarkii* were used. The abdomen was separated from the rest of the animal by a cut between the last pair of walking legs on

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the ventral side and the posterior edge of the carapace dorsally. The isolated abdomen was fastened ventral side uppermost by pins through the first abdominal segment in a dish containing ice-cold crayfish solution. The chain of abdominal ganglia was next exposed by removing a strip of cuticle from the mid-ventral portions of segments 2-5. Care was taken not to damage any of the three pairs of nerves from any of these ganglia. The first pairs of roots are especially vulnerable as they run close beneath the rigid sternum of each segment. The cord was usually prepared for splitting between the third and fourth ganglia by removal of the sternal blood vessel and by placing a small piece of black film beneath it. The sheath surrounding the

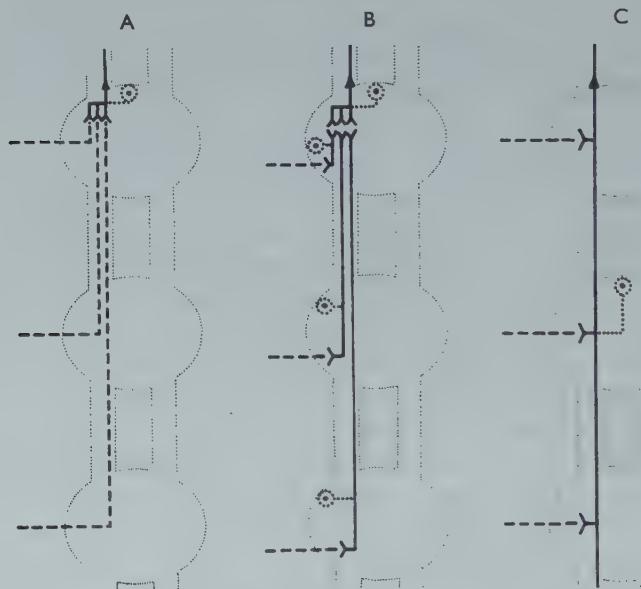


Fig. 1. Diagram to show three possible types of neural connexions which would result in a single interneurone firing when sensory areas of three separate segments were stimulated. Dashed lines show primary sensory fibres; dotted lines indicate the cell bodies of the interneurones, whose locations are unknown; full lines interneurones. Synapses in which several pre-synaptic fibres converge on a single post-synaptic fibre are shown: $\dashv\dashv\dashv\dashv$, whereas a synapse between only two fibres is shown: $\dashv\dashv$. The same conventions are adopted in Figs. 3, 6, 8 and 9. For further explanation see text.

connectives was split horizontally, and this section was extended from just in front of the fourth ganglion to the region where the third roots of the third ganglion leave the cord. As in previous work with the circumoesophageal commissures, these and all subsequent splits were marked on a schematic outline of a cross-section so that the location of a bundle under investigation was approximately known (Wiersma, 1958). Leading off was first done 'biphasically', i.e. by lifting the bundle on a single electrode just above the surface of the fluid which was grounded. Subsequently, depending on the type of response from it, the bundle was further divided or the lead was made monophasic by cutting the bundle and lifting it out of the fluid. After a short drying-out period, monophasic responses were then obtained. The lead was

taken either from the anterior or posterior part of the bundle, or in some instances from both at the same time with the use of a second electrode. In general, only those bundles were used in which the main response gave proof of being definitely due to a single unit on the oscilloscope and through the loud-speaker.

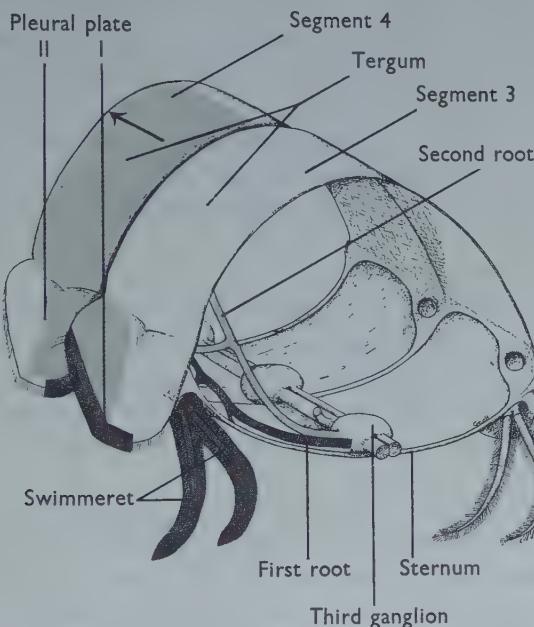


Fig. 2. Stereogram of the third and fourth abdominal segments of a crayfish showing the sensory innervation of the right half of the third ganglion. First root's fields: dark shade; second root's fields: light shade. Arrow indicates the position of the right fast and slow abdominal stretch receptors for the joint between the fifth and fourth segments.

Mechanical stimulation in all experiments in which touch of hairs was investigated was done by touching different parts of the exoskeleton with a fine brush, and if necessary for more precise localization of the stimulus area, by a bristle or needle. Joints were moved by manipulation with needles and sometimes manually or by reflex contractions. Care was taken to localize the stimulus as much as possible to one area or joint. In some cases this was extremely difficult because of the high sensitivity of certain end-organs which responded, for instance, as soon as the meniscus of the fluid was disturbed anywhere in the dish. Nomenclature used to describe the areas which excited a given fibre when stimulated is shown in Fig. 2.

Most preparations were used for periods up to 4 hr. There is a slow but continuous deterioration in such preparations. As in the commissure the primary sensory fibres remained responsive longer than the interneurones, though the latter often persisted for several hours. In most preparations the first abdominal ganglion was unresponsive from the start but the sixth ganglion, although also less exposed than the others, did not obviously differ from them in its reactivity. During the

later stages the interneurones failed first to react to stimuli reaching them through the second ganglion, whereas the other ganglia became affected later all at about the same time.

RESULTS

The units from which recordings were made can be classified into the two major groups of primary sensory fibres and interneurones. In most instances this distinction was easily made, since the majority of primary sensory fibres respond to more localized stimulation, with higher frequencies and with smaller impulses, whereas the interneurones, in addition to being responsive to stimulation of a larger area and having impulses of greater recorded amplitude and lower frequency, often show more rapid fatigue and other 'central' features such as inhibition and facilitation.

(A) Primary sensory fibres

As stated above, these form a far greater portion of the fibres in the abdominal connectives than they do in the circumoesophageal commissures. They enter the cord through the first and second roots of each segment, the third roots being entirely motor (Hardy, 1894; Wiersma, 1947a). The two roots supply quite distinct sensory areas and it is convenient to consider each of them with its central pathways separately.

(i) *First root areas.* Sensory fibres from hairs and joints of the swimmerets enter via this root, together with hair fibres from the ventral abdominal surface as well as those from the distinct tuft of hairs on the lateral edge of each segment (pleural plate I, Fig. 2). In this figure it can be seen that all fibres from a given swimmeret enter the ganglion situated in that segment, but that the sensory inflow from hairs of the anterior third of pleural plate I enter the ganglion next to the anterior. In the cord between the third and fourth ganglia, primary fibres ascending from the fourth and fifth segments and descending from the third segment are found which respond to movements of the swimmeret joints. These are of various types depending for activation on the direction of movement and differing also in rate of adaptation. They are not restricted to basal joint movements but are also found for the more distal joints of the swimmerets. In some cases the nature of the discharge which occurred mainly or only during active movements suggested that they might be due to the type of ending described by Alexandrowicz (1958) in the coxal region as 'muscular receptors'. All sensory fibres appear to form distinct bundles, in which those of similar origin run together. There is definite evidence that the bundle of swimmeret joint fibres ascend through at least two ganglia, but they do not seem to descend through more than one. The bundles of primary hair fibres from the swimmerets and from the pleural plates spread much less from their place of entry into the cord, solely descending to the next ganglion.

(ii) *Second root areas.* Through this root enter the well-known large primary fibres from the stretch receptors in the abdomen. These have also been found in the circumoesophageal commissures. They are recognizable by their characteristic discharge on flexion of individual abdominal joints. Any doubt that might exist about

their primary nature once they have entered the cord could be removed in the present experiments by recording simultaneously from the second root and from the fibre dissected in the abdominal cord. Identical records except for a small difference in the arrival times were obtained. Their conduction speed is about 8 m./sec.

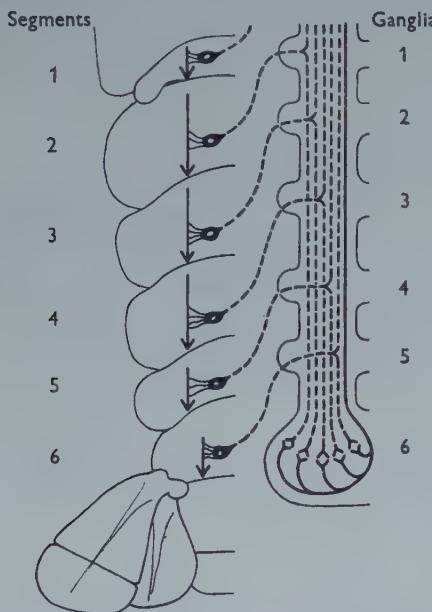


Fig. 3. Diagram of the path of sensory fibres from the homolateral slowly adapting muscle receptor organs in the abdomen. One half of the cord, from the first to the sixth ganglion, is shown. Primary sensory fibres are indicated by dashed lines, the interneurone of the sixth segment by a solid line (see text). Arrows indicate the joint which when flexed excites a given receptor.

Two unexpected features were established concerning the course of these fibres. In the first place, the second root in which they enter the cord is one segment more anterior to the segment whose posterior joint they serve. For example, fibres from the sense organs signalling flexion of the hinge between the fifth and the fourth abdominal segments enter via the second root of the *third* abdominal ganglion (Figs. 2, 3). Secondly, not only do they send out an anterior branch as is necessary to account for their presence in the commissure, but they also have a posterior branch which ends in the sixth ganglion. Thus it is possible to lead off impulses both in front and behind a given ganglion, which are again identical with respect to frequency. These experiments clearly demonstrate that transmission at their T (or Y) junctions in the ganglion is equal in both directions. As expected, it was found that these responses are completely homolateral, the fibres running up and down the cord at the same side at which they enter (Fig. 3). From a few experiments in which the two fibres of the slowly adapting organs of one segment were both prepared, it was evident that the responses were very much alike in overall frequency but were not identical in their detailed pattern. The central branches of the stretch

receptor fibres form a very definite bundle, both in the commissure and in the cord. In the latter the ascending and descending branches are mixed. This bundle is located ventral of the median giant fibre in both places.

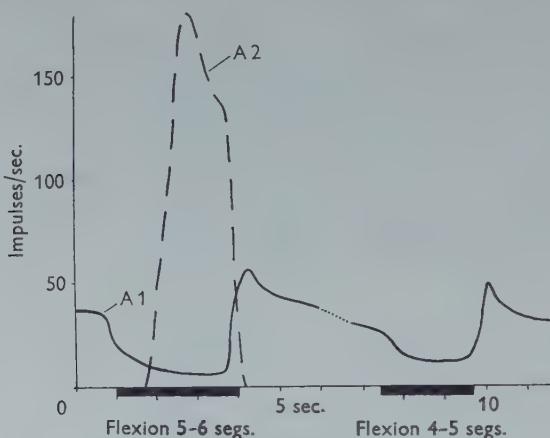


Fig. 4. Plot of impulse frequency in two fibres of an abdominal connective between the third and fourth ganglion during flexion of: (a) joint between fifth and sixth segments, (b) joint between fourth and fifth segments. A 1, tonic extensor fibre; A 2, slowly adapting fibre of the joint between the sixth and fifth segments.

In this same bundle other fibres were found which were stimulated by the opposite movement, i.e. tail extension. One of these (A 1 of Fig. 4) discharges tonically even when the tail is in the position which it takes up when no forces act on it other than the very mild one of gravity on the unsupported telson. By leading off at the same time from one of the tonic stretch receptors (Fig. 4, A 2) it can be shown that for flexion of this segment there is a reciprocal relation of the discharges; flexion of another segment will, of course, not affect the same stretch receptor, but does have a similar inhibiting effect on the extensor fibre. In correspondence with this latter finding, the frequency of the extensor response can be increased by extension of any of the joints between posterior segments. Complete and lasting stopping of firing occurs only when all of them are kept flexed. Many attempts were made to locate the sense organs involved in these discharges. All roots of different abdominal ganglia have been investigated, but in none of them were such signals found, and in the cord they persisted when all first, second or third roots were cut, also in combination. It is known (Wiersma *et al.* 1955) that in the commissures certain fibres may be brought to discharge by stretching the axon with the leading-off electrode. By preparing long lengths of the cord fibres, which allowed for slack even when the fully stretched position of the tail was reached, this possibility was here excluded, the response taking place as readily in the slack nerve fibre. But this does not exclude the possibility that stretch of the axon is the stimulating factor in the sheathed part of the cord. Another possibility is that these fibres have dendritic processes in the cord sheath and signal its stretching. Whether the fibres themselves are primary or

secondary is unknown, though they do signal stretch of more than one segment. It should be stressed that there are three such fibres, which differ in their properties, one being phasic, one tonic and one intermediate, and that all three have been found several times in the same preparation. This constancy of response patterns and the constancy in their location make it highly likely that the perception of abdominal stretch is their true function and not an artifact.

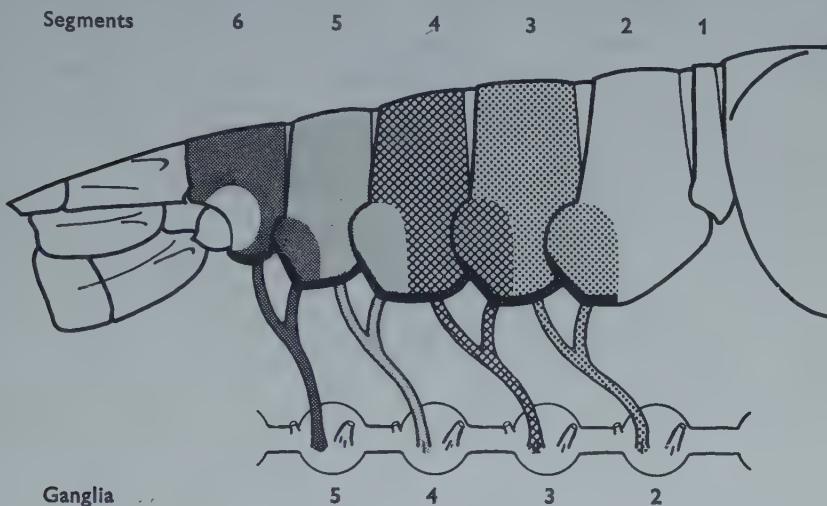


Fig. 5. Diagram to show the peripheral pathways of sensory fibres from hairs on dorsal abdominal segments which enter through the second roots of the second to fifth abdominal ganglia. Note the slant of the neural segment as compared to the skeletal one.

The hair fibres from the sensory areas on the dorsal side which enter through the second root are also mostly from the segment posterior to that containing the ganglion. As shown in Figs. 2 and 5, the sensory input from the same segment comes only from a small part of the posterior pleural region, whereas that from the next posterior segment comes from the whole dorsal area except for a corresponding pleural area. In the cord such fibres form broad bundles in which those of adjoining segments are associated. They are both ascending and descending. Whether or not the descending fibres are all only from the dorsal area of the posterior segment and not from the posterior lateral area of the same segment is not known.

(B) Interneurones

As in the commissures, interneurones were found which reacted either to touch or to proprioceptive stimuli and a few to both. Depending on whether the stimulation was effective on the same side of the body in which the interneurones run, or on the other side or both, they can be distinguished as homolateral, heterolateral or bilateral interneurones.* In each of these classes, interneurones are present which react on stimulation of a single abdominal segment or of several consecutive segments.

* Some asymmetrical fibres which include both homolateral and heterolateral areas, but not symmetrically divided, are also present, but will not be further discussed here.

Quite a number of fibres have been found which apparently only respond to stimulation of dorsal hairs on a single segment, which had not been encountered in the circumoesophageal commissure. Most of these are homolateral but similar heterolateral fibres have also been obtained. In contrast to the primary sensory fibres, responses can be obtained by stimulating quite different places on the dorsal surface and they have a distinctly higher 'threshold', e.g. do not readily fire with a slight touch of a brush and show a much lower frequency of response. Interneurones excited by afferents in the first root of a single segment have also been found. It is difficult, however, to distinguish those which react to proprioceptive

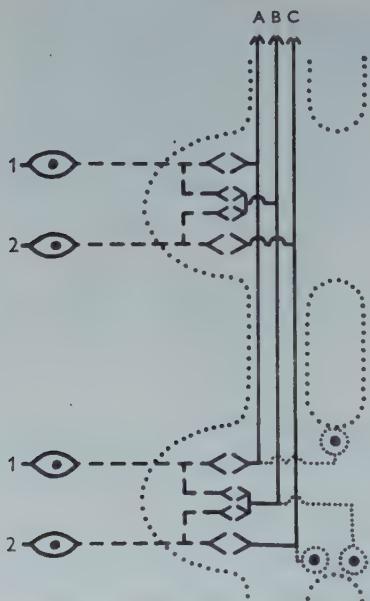


Fig. 6. Diagram of three different interneurones, two of which (A, C) respond to stimulation of two distinct types of sensory field (1, 2) on several segments, whereas type B is excited by stimulation of both of the areas which A and C innervate.

function of the swimmerets from the similar primary sensory fibres, since the latter may also give rather low frequency responses, and 'areal' stimulation is almost impossible because of the structure of the swimmeret. Interneurones responding to only two segments, which are then always consecutive, are present for both homolateral dorsal and heterolateral dorsal areas, but not for ventral ones. There are also some very good examples of fibres responding to stimulation of three or more dorsal segments as well as for fibres responding to several ventral segments or to both dorsal and ventral parts combined. Several of these are identical in their properties with those found in the commissure.

Like single-segment interneurones, those responding to several segments may do so only to a specific part of each segment, for instance, the hairs on pleural plate I. A fibre responding to two segments in this way is represented by 'A' in Fig. 6.

Fibre 'C' represents a similar one of this type, e.g. one responding to hairs on the swimmerets. In addition to these fibres which collect more detailed information, fibres are present which are connected like 'B', in this example thus sensitive to hairs on both the pleural plate I and the swimmeret.

The fibres responding to homolateral sensory fields in three or more segments offer the best opportunity to test for the three possible arrangements shown in Fig. 1. In all of these interneurones tested with a 'diphasic' lead in the connective between the third and fourth ganglion, it was found that impulses passed in both directions when the extent of their sensory fields was such that this was to be expected. Descending impulses could be observed on stimulation of anterior fields, ascending ones on posterior stimulation. After cutting and applying a lead to each end, the two leads only record impulses from the appropriate fields. It is essential, of course, to know that all reactions do take place in the same interneurone. In the 'diphasic' lead this is often very clear at the beginning of an experiment, whereas in later stages there may develop a difference in shape between descending and ascending impulses, due to a block in the area of the electrode. We have therefore used other ways of recording as well, in order to substantiate this observation. As collision of impulses must occur, 'diphasic' recordings were made from the same small bundle with two electrodes at about 6 mm. distance. The direction of the impulses was then clearly indicated by the lead in which they first appeared. Again, unequivocal evidence was found in many preparations that impulses pass in both directions according to the areas stimulated. With simultaneous stimulation of areas, evoking impulses travelling in different directions, it has, in a few cases, been possible to prove that when two impulses arrive simultaneously at the two electrodes, neither of these reaches the other electrode, proving that they, in contrast to all others, collided *between* the electrodes (Fig. 7). This type of observation has been made for interneurones excited by sensory inflow from either first or second roots, but for many purposes those excited by the joints of all homolateral swimmerets were the most convenient as the stimulation was more easily localized and the discharges in some of these fibres were distinctly tonic.

In contrast to these homolateral interneurones, some of the bilateral ones show the interesting feature that after cutting them between the third and fourth ganglia they still respond in the anterior recording to all the homolateral and heterolateral sensory fields to which they were sensitive in the 'biphasic' recording, and thus to segments of the abdomen both in front and behind the leading-off position. Furthermore, this can also obtain for the posterior lead. These observations make it necessary to postulate central connexions of the type D shown in Fig. 8. For such a fibre it is necessary to suppose that it makes connexion with its homologue on the other side in each ganglion, a situation known to exist for the lateral giant fibres (Wiersma, 1947a; Furshpan & Potter, 1959). If these connexions are fairly labile, different types of results are to be expected depending on which junctions fail to transmit. Such variations have been found between fibres which were identical in their location and sensitive fields. A special case of this is shown in scheme E of Fig. 8, for which there is evidence that it may occur naturally in a special fibre, but in other

cases was apparently due to the loss of junctional transmission in all but the most anterior cross-connexions. This type E pathway explains the cases, which have been rather frequently encountered, where the fibre only responds on the homolateral side behind the cut in the posterior lead, whereas the whole remaining part of the receptive field including the posterior heterolateral areas produce responses in the anterior lead. Note that on the homolateral side such interneurones are excited directly by sensory fibres, but on the heterolateral side by an interneurone.

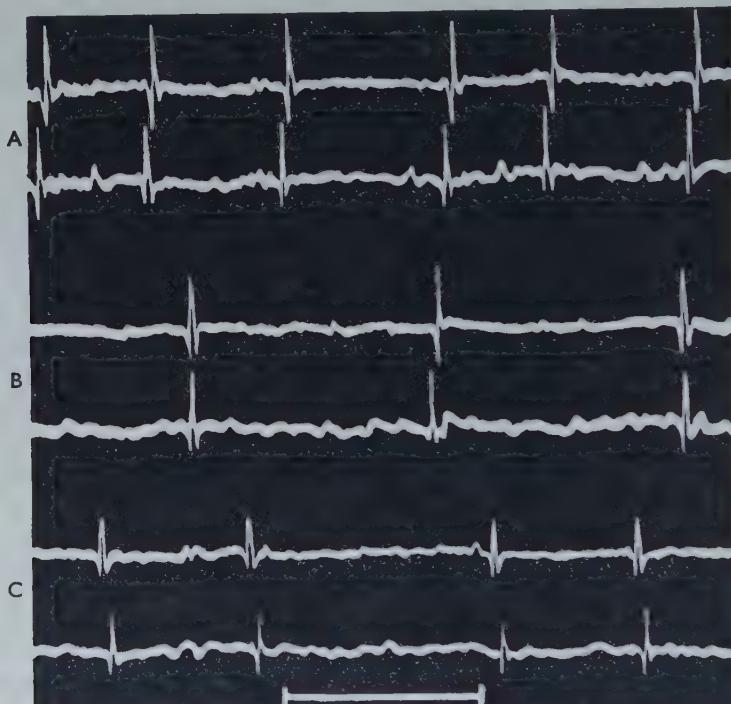


Fig. 7. Three records from two 'diphasic' leads from a small bundle of fibres in a 3-4 abdominal connective. In each frame the upper of the two lines is from the anterior lead. Stimulation in upper frame from posterior segment, in lower frame from anterior segment, and in middle frame from simultaneous stimulation. The first and last impulses in the middle frame collide between the leads. Cuts from long records. Note the gradual reduction of action potential size between frames which developed with time. The spikes are retouched. Time $\frac{1}{60}$ sec.

Some other bilateral fibres do, however, invariably respond in a way analogous to that described above for homolateral interneurones, being excited in the anterior lead only by the bilateral sensory fields above, and in the posterior ones by those below the lead. It is here necessary to postulate a pair of interneurones which do not make mutual synaptic connexions, but which in each ganglion are stimulated by sensory fibres from both sides of the body (scheme F of Fig. 8). There are two alternatives with regard to the way in which crossing in the ganglia occurs. Either primary sensory fibres from the heterolateral side may decussate to make connexion

with the interneurone, or the latter may send a collateral to the neuropile of the other side. From what is known of the histology of crustacean ganglia both alternatives are possible.

This same question arises with regard to the heterolateral interneurones, several of which are found to be responsive to stimulation of dorsal hairs of successive abdominal segments. In most of these the impulses appear to arise in either one or the other side of the lead, and are thus explained by scheme G of Fig. 9. However, it was found that a fibre for the fifth heterolateral dorsal segment gave descending as well as ascending impulses in a lead between the second and third ganglia. This indicates connexions of the type shown in scheme H of Fig. 9, in which the heterolateral unit is a secondary interneurone.

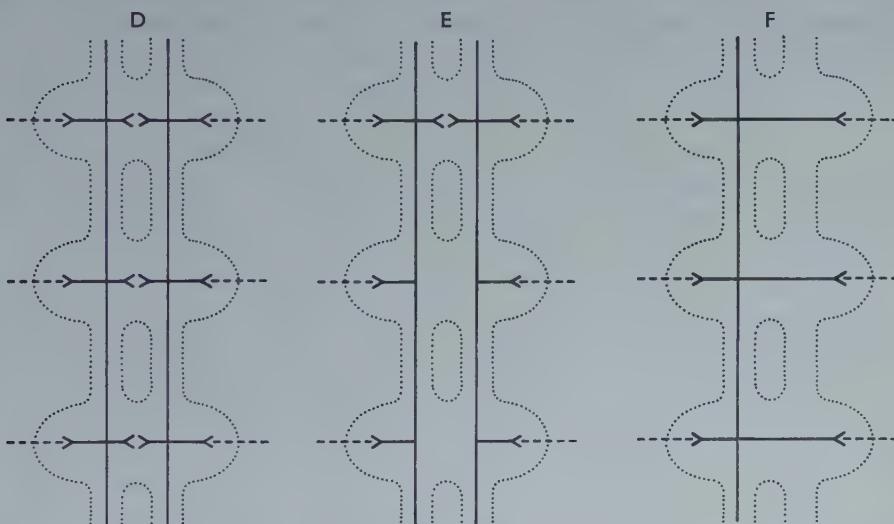


Fig. 8. Three different types of connexions (D, E, F) indicated by the findings in interneurones responding to bilateral sensory stimulation. For explanation see text.

The behaviour of all interneurones discussed above which respond to stimulation of receptive areas on three or more segments, whether these be homolateral, bilateral or heterolateral, strongly suggests that without exception they can be excited in each ganglion and are thus of type C (Fig. 1). But this is not the only type realized, for clear evidence has been found for type A (Fig. 1) connexion in at least one interneurone. This fibre integrates the inputs from all of the primary sensory fibres of the slowly adapting muscle receptor organs on one side of the abdomen. (This, strictly speaking, is not proven, as the most anterior ones of these, abd.segm. 1-thorax, and abd.segm. 2-1 could not be tested in the preparations made.) As can be seen in Fig. 3, the region where this integration occurs is in the last (sixth) ganglion and the fibre is thus activated by descending impulses in contrast with the situation pictured in Fig. 1A. The final experiment on which this fibre was established as a unit in the cord was made possible only because of the previously acquired know-

ledge of the primary sensory pathways. In this experiment the fibre, integrating all tonic stretch receptors, was found at a time when all the descending branches of the latter were still intact. The interneurone was found to respond therefore as expected, to both flexion of the fifth on the fourth abdominal segment, as well as to that of the sixth segment on the fifth. By cutting the bundle containing the primary sensory fibres in the connective between the third and fourth ganglia, it would follow that the reaction to the first-mentioned stimulus should disappear (since the stretch receptor axon enters the second root of the third ganglion) but that the other should remain. This actually was found, and since the heterolateral connective was intact, it also followed that the interneurone was a homolateral one. These experiments also indicate that the integration must take place either in the fifth or the sixth ganglion. The latter appears the much more likely possibility, as histological evidence indicates that the descending branches of the primary muscle receptor fibres do not stop in the fifth but run through to the sixth ganglion.

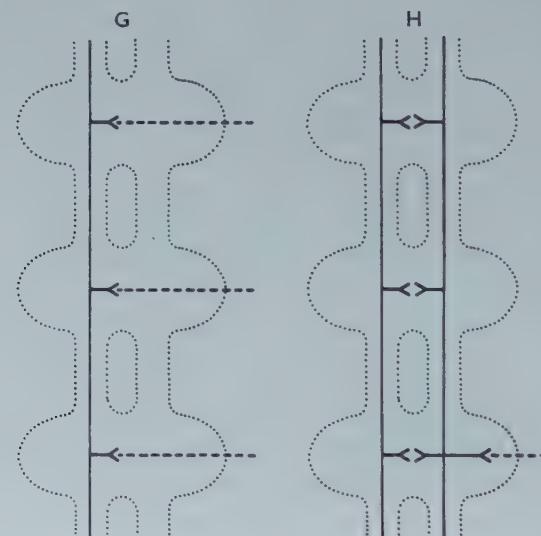


Fig. 9. Two types of connexions, (G, H) of heterolaterally responding pluri-segmental interneurones, as indicated by experimental observation. For explanation see text.

Direct evidence for the existence of type B (Fig. 1) pathways has not been obtained in the present work, but its possible presence is indicated by the finding of many interneurones which respond to sensory areas of only one segment. Such fibres were not found, except for the first abdominal segment, in the commissure. It is therefore possible that some of these lead to integration in a more anterior ganglion and form some of those interneurones in the esophageal commissure which, in contrast to others, have not been found in the cord.

DISCUSSION

The methods used in the present work have given useful information not only concerning the types of synaptic connexions which exist between different primary sensory fibres and interneurones, as presented in this paper, but also concerning the functions and pathways of a number of individual neurones, which will be presented elsewhere. For both aspects histological data are complementary but unable to answer the pertinent questions by themselves. For the present purpose it should be noted that it appears impossible with available techniques to trace the connexions which primary sensory fibres make with specific interneurones in the neuropile. From our results it is quite clear that such connexions follow very definite rules, e.g. one interneurone making connexions with one part of the sensory inflow, another with a different part, and a third with both (Fig. 6). This type of finding requires the precise development of all these connexions, a picture greatly different from that which is obtained from the histological one in which there appears little of this type of order in the neuropile.

Whereas in this respect histological findings are as yet of no help, in other cases they complement the physiological results and confirm the presence of certain elements at an anatomical level. For instance, in Allen's Figs. 11 and 12 (1894), rather large fibres are shown which enter the second root of an abdominal ganglion in the lobster and then form a T-junction with ascending and descending portions, but without a branch into the ganglion it enters. Such a fibre is exactly of the sort that would explain the functional pathway found for the primary sensory fibres of the abdominal muscle receptor organs. Furthermore, Allen's observations on the embryonic lobster have been confirmed and extended by Alexandrowicz (1951) who was able to show that these fibres arose from peripheral cells which are almost certainly precursors of the muscle receptor organs. Our results, made independently and by another technique, completely confirm Alexandrowicz's finding that these fibres form a distinct tract which lies ventral and medial to the medial giant fibre in the connectives. All these observations indicate that such fibres do not have a local reflex arc within the abdominal ganglia, but would excite structures in the upper and lower portions of the central nervous system. For the latter we have direct evidence by finding an interneurone which is excited by all slowly adapting organs, whereas the absence of interneurones stimulated by these fibres in the abdominal ganglia (2-5) may support the idea that they do not make connexions within their neuropiles.

Again, Retzius (1890) shows primary sensory fibres which enter by the second root and immediately descend to another ganglion, and we obtained responses from primary sensory hair fibres of the dorsal abdominal segment which descended. Previously most primary sensory fibres of the roots do not appear to have been traced to their origin. As has been shown, the roots do not innervate the periphery according to the external segmental divisions. Instead, the neural segment slants in a dorso-posterior direction (Fig. 5). The motor axons in the second root innervating the dorsal extensor musculature show a similar distribution (Hardy, 1894).

Hardy considered that displacement of the dorsal muscles is the reason for the shift, and noticed that the blood supply does not take part in it. From our observations on the sensory innervation it is clear that the whole neurotome participates; the motor fibres of the dorsal muscles to a greater extent, since they penetrate into the next posterior segment (Wiersma 1947*b*).

Histological information concerning the different types of interneurones is more difficult to interpret because here practically all quantitative data about the numbers present of the different types described are lacking. Bethe (1897), however, has noted the presence of several interneurones which have connexions similar to those suggested diagrammatically in this paper, including some bilateral fibres of type F (Fig. 8). He also shows fibres which are essentially type E but the transverse connexion between the two sides is asynaptic. These and some unilateral interneurones have segmentally arranged synaptic sites, a type for which there is now abundance of physiological evidence. The existence of this type of connexion (scheme C of Fig. 1) is one of the main conclusions of the present work, but as stated above, types A and B certainly cannot be excluded. It is of interest to consider the different physiological properties to be expected from these three systems and the functions they might serve in the central nervous system.

Type A is the normal one considered to account for the integrative action of interneurones excited by sensory stimulation. In this case it is the properties of the synaptic connexion which will determine how this integration takes place. Variations of threshold and excitability in the synaptic regions will result in interneurones with quite different properties, such as those very sensitive to a few sensory impulses but with quick adaptation, and those which fire at low constant rates to low frequency inputs. During our investigation we have noticed very significant differences between interneurones in these respects (see also, Preston & Kennedy, 1958). In addition the picture can be complicated by the possibility of inhibitory innervation at the same locus. In the abdomen very few fibres have been found which clearly require such a mechanism, though the possibility that it is more widespread is not excluded. The interneurones involved were stimulated either by sensory fibres from hairs or joint organs. The evidence from the former is the more striking. For such fibres, in sharp contrast to most others, the mapping of the sensory area, limited to one segment, is quite easy because touch of surrounding areas inhibits any low frequency discharge caused by slight stimulation of the area.

In type B integration the difference from type A is that two synapses have to be passed. Depending on the synaptic properties of these two, the output in the secondary interneurone could differ very considerably between different interneurones. This arrangement would seem particularly suited for integration of different modalities, but there is no evidence that this is the case. On the contrary, the interneurones known to respond to different modalities appear to be primary interneurones making synaptic connexion with the different kinds of primary sensory fibres.

Some of the properties of connexions of type C have been partially discussed (Wiersma, 1958). Obviously the 'local sign aspect' of the stimulus is increasingly

reduced when additional numbers of segments are involved. Since several types of axons are found in this class, differing in the same way as pointed out for type A interneurones, the consequences for impulse transmission to the 'receiving higher centre' will be considerably different for these reasons. In the type of axon which fires at low frequencies only, the summation aspects will preponderate. For if in the different ganglia only low-frequency impulses are set up, the chances that they are blocked by 'antidromic' impulses diminishes. Of course, the conduction speed of the impulses and the distance between the synaptic areas are also involved. On the other hand, in those interneurones which regularly carry high-frequency signals, occlusion of almost all of the impulses from the receptive areas farthest removed from the 'receiving centre' must be a regular occurrence during simultaneous stimulation. If the centre be the brain, it would follow that more impulses would reach it when quick successive stimulation took place from back to front than from front to back, since it would always be the area closest to the receiving centre which would have the best chance to 'squeeze' impulses in the trains coming from the remoter ones.

There is as yet no evidence whether the type of integration found in interneurones which collect in different ganglia can also occur in the branches of such interneurones in single ganglia. Interneurones are present which collect from more than one of the neuropiles of a single ganglion (Allen, 1894; Bethe, 1897), which may indicate that there is also within a ganglion more than one site at which action potentials can be generated. This arrangement will cause collision of impulses instead of summation of prepotentials. A considerable strengthening of this possibility may be seen in the fact that with internal electrodes two spikes of different shape have been reported to occur in single cells of the sixth ganglion (Preston & Kennedy, 1958). Bullock & Terzuolo (1957) have recorded independent action potentials in different parts of crustacean heart ganglion cells, showing that, here too, more than one locus for spike generation is present in a single cell. It is interesting to note that in this type of system, slight shifts in position of the site of spike generation could change its properties considerably. If this site is close to the neuropiles, each would be able to set up spikes which would mutually collide. But if the site were further removed, the local potentials of the neuropiles would summate and the frequency of spikes would be proportional to the excitation of both combined. The long distances between ganglia precludes the latter mechanism from playing more than a minor part in interganglionic integration.

From a comparative point of view it is likely that the three types of interneuronal connexions discussed here exist in many central nervous systems. In practically all groups of animals histological evidence is available for the presence of interneurones having synaptic regions in spatially separated neuropiles. Although such pathways could function in a variety of ways, it is probable that in many instances the properties described for type C connexions in the crayfish must be considered when assessing their mode of action.

SUMMARY

1. An investigation has been made into the function and distribution of nerve fibres in the abdominal ganglion chain and its roots in the crayfish, *Procambarus clarkii*, by leading off action potentials from small prepared bundles following sensory stimulation.

2. The sensory fields belonging to the first and second roots of each abdominal ganglion were determined, and the antero-posterior pathway of sensory fibres within the cord noted. It was found that the primary sensory fibres of the dorsal muscle receptor organs, entering through the second root, send out an anterior branch to the brain and a posterior one to the last ganglion. For most other sensory fibres much shorter intracentral branches are indicated, though some of them extend for two ganglia in the anterior direction and for one posteriorly. All sensory fibres in the connectives run on the same side as they enter.

3. The segmental divisions of the external skeleton and of the nervous system do not coincide, the neural segment slants in a posterior dorsal direction with respect to the skeletal one.

4. For the majority of the interneurones which innervate more than two abdominal segments it has been proved that they synapse with primary sensory fibres in each of the ganglia that these enter. Depending on the segment stimulated with respect to the leading-off position, both ascending and descending impulses are obtained in such interneurones and collision of the impulses has been observed. Some consequences of this type of integration are discussed.

5. For interneurones responding to bilateral or heterolateral stimulation the course of the impulses proved to be of at least two types. In some, cutting the fibre prevents the arrival of impulses except those set up on the side of the cut from which the recording is made. In others, recording from either side of the cut fibre does not exclude any of the sensory fields to which the fibre normally responded.

6. At least one interneurone is present in which all primary sensory fibres from the different segments to whose activity it responds collect in one ganglion.

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A STUDY ON THE FIBRE DIAMETER AND CERTAIN
ENZYME CONCENTRATIONS IN THE FLIGHT
MUSCLES OF SOME BUTTERFLIES

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INTRODUCTION

Recent studies in insect metabolism indicate that, in certain insects like the beet-leaf hopper, locust and some Lepidoptera which indulge in sustained flight, fat forms the chief fuel (Fulton & Romney, 1940; Krogh & Weis-Fogh, 1951; Weis-Fogh, 1952; Zebe, 1954). In some cases it has been found that a respiratory quotient around 0·75, which is indicative of fat utilization, was obtained even at rest and in the presence of considerable amounts of glucose (Zebe, 1954, 1959). Flying vertebrates like birds and bats are also believed to utilize fat as the chief source of energy during sustained muscular activity (George & Jyoti, 1955, 1957, 1958; Odum & Connel, 1956). In this context the discovery of high concentrations of a fat-splitting enzyme (lipase) in muscles capable of such prolonged activity such as pigeon and bat breast muscles (George & Scaria, 1956; George, Susheela & Scaria, 1958a), locust and dragon fly flight muscles (George, Vallyathan & Scaria, 1958) and also cardiac muscle (George & Scaria, 1957) is of special significance.

The results of the recent studies conducted in our laboratories on the flight muscles of pigeon and bat have indicated a close relationship between structure and function. It has been shown that in both pigeon and bat breast muscles there exist two different types of fibres, dissimilar in size, colour, content of mitochondria, enzymes and metabolites (George & Naik, 1957, 1958, 1959; George & Scaria, 1958a, c; George, Susheela & Scaria, 1958a, b). In these muscles a correlation could be established between the concentration of the different enzymes and their fibre diameter (George & Scaria, 1958a, b, c; George, Susheela & Scaria, 1958a, b). A similar correlation was also arrived at in the skeletal muscles of the rat by Nachmias & Padykula (1958). Oxidative enzymes are concentrated more in fibres with lesser diameter and as a rule highly active muscles have more narrow fibres. Tiegs (1955) studied the anatomy and histology of the flight muscles of a large number of insects. However, he did not include in his study the fibres of the flight muscles of butterflies. A study on the flight muscles of butterflies should be of considerable interest since some butterflies fly short distances while others

indulge in long and sustained migratory flights. Here we report our observations on the fibre diameter and the concentration of enzymes, especially lipase, in the flight muscles of a few butterflies in an attempt to correlate structure and function at the cellular level.

MATERIAL AND METHOD

The flight muscles of the butterflies belonging to the following four families were studied:

Family	Name
Danaidae	<i>Danais chrysippus</i> (Linnaeus)
	<i>Euploea core</i> (Cramer)
	<i>Danais limniace</i> (Cramer)
Nymphalidae	<i>Hypolimnas bolina</i> (Linnaeus)
	<i>Precis lemonias</i> (Linnaeus)
Pieridae	<i>Dalias eucaris</i> (Drury)
Papilionidae	<i>Papilio polytes</i> (Linnaeus)
	<i>Zetidus agamemnon</i> (Linnaeus)

They were captured during flight and used immediately, after killing by decapitation. The thorax was opened on the ventral side, and after pulling out the alimentary canal the muscles were carefully removed from their attachment by means of a pair of clean forceps and used for the various studies.

Fibre diameter

Thin hand sections of fresh frozen muscles, cut according to the method of George & Scaria (1958a) and mounted in glycerine jelly were used for the measurement of fibre diameter. A microscope with an ocular micrometer was used for the measurement.

Enzymes

(i) Quantitative

The lipase concentration in the flight muscles of all the above butterflies was studied quantitatively in a manometric system in a bicarbonate-carbon dioxide buffer of pH 7.4 at 37° C., using tributyrin as substrate as described in an earlier publication (George, Vallyathan & Scaria, 1958). Lipase activity is expressed as the amount of $\mu\text{l. CO}_2/\text{mg. protein/hr.}$ Protein was estimated according to the micro-Kjeldahl steam distillation method (Hawk, Oser & Summerson, 1954).

(ii) Histochemical

The presence and localization of the following enzymes were studied histochemically: lipase, acid and alkaline phosphatases, adenosine triphosphatase (ATPase) and succinic dehydrogenase.

For histochemical study of the enzymes fresh frozen sections of the muscles, prepared according to the method described by George & Scaria (1958a), were used. Lipase was studied according to the method of Gomori (1953) using 'Tween 80' as substrate at pH 8.4 (George & Scaria, 1958a). The revised method of Gomori, using sodium glycerophosphate as substrate, was employed in the study of acid and alkaline phosphatases (George, Nair & Scaria, 1958) at pH 5.0 and 9.2

respectively. Sections kept in boiling water for 10 min. and incubated along with the samples under investigation were taken as control. The sections were incubated for 6–8 hr. for lipase and for 6 and 24 hr. respectively for acid and alkaline phosphatases at 40° C. The method of Pearse and Reis (Pearse, 1954) was adopted for the demonstration of ATPase. Sections kept in the incubation media for alkaline phosphatase at pH 7.4 and 9.2 were used as control. The period of incubation was 3 hr.

Succinic dehydrogenase activity in the muscle was determined by the tetrazolium chloride reduction method exactly as described in an earlier paper (George & Scaria, 1958c). 2:3:5-triphenyl tetrazolium chloride was used as the electron acceptor.

RESULTS

(1) Measurement of fibre diameter

The average diameter of the fibres in the different species is given in Table 1. It was observed that the diameter of the fibres in the muscle was more or less uniform for the species. Fibres with the largest diameter (130–178 µ) were found in the Papilionidae and the smallest in the Danaidae (80–98 µ). The Nymphalidae and Pieridae have fibres ranging from 84 to 105 µ in diameter. The figures given are the average of several hundred fibres from thirty to forty animals.

(2) Lipase activity of the muscles

The results of the quantitative determination of lipase activity in the flight muscles of the different species is presented in Table 1. It can be seen that the greatest activity of 22.92 µl. CO₂/mg. protein/hr. is in the danaid butterfly, *Danais chrysippus*, which has the narrowest fibres and the least (4.52 µl. CO₂/mg. protein/hr.) in the papilionid, *Zetidus agamemnon*, with the largest muscle fibres.

Table 1. *The lipase value* of the flight muscles of different butterflies and the average diameter of their muscle fibres*

Family	Name	Lipase activity (µl. CO ₂ /mg. protein/hr.)	Average fibre diameter (µ)
Danaidae	<i>Danais chrysippus</i> (L.)	22.92	80
	<i>Euploea core</i> (C.)	13.44	90
	<i>Danais limniace</i> (C.)	11.65	98
Nymphalidae	<i>Hypolimnas bolina</i> (L.)	16.37	84
	<i>Precis lemonias</i> (L.)	7.10	105
Pieridae	<i>Delias eucaris</i> (D.)	11.42	95
Papilionidae	<i>Papilio polytes</i> (L.)	5.67	130
	<i>Zetidus agamemnon</i> (L.)	4.52	178

* The lipase value given in the table is the average of five experiments.

(3) Histochemical observations

Lipase

The flight muscles of all the butterflies studied except the papilionids (*Papilio polytes* and *Zetidus agamemnon*) gave positive staining reaction for lipase. The distribution of the enzyme was uniform throughout the muscle fasciculi. However,

from the histochemical observations no definite conclusions could be arrived at regarding the quantitative differences in the flight muscles of the different butterflies (Pls. 4, 5, figs. 1-8).

Phosphatases

All the phosphatases tested for, viz. acid and alkaline phosphatases and adenosine triphosphatase, could be demonstrated in the flight muscles of all these butterflies. The distribution of these enzymes was also uniform in the fibres. Very strong positive reaction, especially for ATPase, was obtained in all the cases.

Succinic dehydrogenase

The colour developed due to formazan was uniform in the fibres of the same muscle. It was not possible to decide whether there were any quantitative differences in the enzyme content of the muscle of the different butterflies studied.

DISCUSSION

The peculiar type of striated muscle in insects has been of special interest to physiologists and biochemists ever since Von-Siebold, in 1848, revealed the histological features of the striated muscles of insects. Yet not much is known about its physiology. This prompted Chadwick (1953a) to make the remark that 'the flight muscles of insects are among the most specialized contractile tissues in the animal kingdom and at the same time among the least investigated from the physiological point of view'. The recent studies of Tiegs (1955) on the anatomy and histology of the flight muscles of a number of insects have been a valuable contribution, but no information is given about the flight muscles of butterflies. It has been reported that some butterflies cover hundreds of miles at a stretch during flight, and they are in the air for much longer periods than most other insects observed (Chadwick, 1953b). Butterflies therefore seem to be excellent material for studies directed towards a clearer understanding of the physiology of sustained muscular activity.

Insect muscle as a rule is devoid of myoglobin, and its absence is compensated for by the copious supply of oxygen from the tracheal system, the tracheoles penetrating even the individual muscle fibres. As such there is no differentiation into red and white fibres as seen in some vertebrate skeletal muscles. Red fibres in vertebrate muscles are usually narrower than the white ones, and are equipped with a more efficient system of oxidative enzymes. Highly active muscles such as the flight muscles are made up mostly if not completely of narrow fibres. Although no such differentiation of the fibres into red and white occurs in the insect flight muscles it seems that the fibres in the flight muscles of the more active insects are narrower than those of the less active forms. The danaid butterflies are very good fliers. Though they fly slowly and in a relaxed way, they fly for hours continuously without stop. The three danaid butterflies we have studied are all reported to be migratory (Wynter-Blyth, 1957). Among the nymphalids, *Hypolimnas bolina* is a migratory form. Nymphalids are almost similar in their mode of flight to the danaids. In these forms the diameter of the muscle fibre is decidedly less than that

in the papilionids (Table 1), which are supposed to fly fast but only for a short time, indicating clearly that the decrease in diameter of the fibres has some relation to the ability to maintain continuous flight.

The data presented in Table 1 lend further support to the view that a muscle to become more active and efficient should possess fibres smaller in diameter. This view seems to be gaining ground, particularly in the case of vertebrate skeletal muscles indulging in sustained activity. Why it is so in the vertebrate skeletal muscles could be easily explained when we consider that the oxygen supply from the blood to the interior of the fibres is greater and faster when the volume of the individual fibre is less, and thereby the overall surface area of the fibres comparatively much greater. But why it should be so in the case of the insect muscle in which the oxygen supply is by diffusion across the tracheal capillaries which penetrate the fibre itself is not understood. A study of the distribution of the intracellular tracheae in these fibres having different diameters should therefore throw some light on this aspect.

It has been shown by George & Scaria (1956, 1957, 1959) that the amount of lipase present in a muscle is an indirect indication of the extent to which fat could be metabolized in the muscle. In vertebrates the correlation between the concentration of lipase in a muscle and its activity is evident from their data. The same could hold good for the insect flight muscles also (George, Vallyathan & Scaria, 1958). The data presented in this paper is further evidence in favour of their suggestion. Thus the highest value of $22.92 \mu\text{l. CO}_2/\text{mg. protein/hr.}$ is obtained for the (migratory form) *Danais chrysippus* and the lowest of $4.52 \mu\text{l. CO}_2/\text{mg. protein/hr.}$ for the papilionid, *Zetidus agamemnon*, which is a comparatively poor flier. Further, it can also be seen that in the other butterflies studied the lipase value varies more or less according to the diameter of the muscle fibre. These observations speak unmistakably in favour of the postulated relationship between the diameter of the fibres in muscle and their enzyme concentration and activity. Again, the higher concentration of lipase in the more active muscle suggests the utilization of fat for energy during flight. As already pointed out, in the desert locust (*Schistocerca gregaria*) it has been shown that fat is the chief source of energy for flight. Beall's study (1948) on the fat content of the monarch butterfly (*Danais plexippus*) during migration, indicates the utilization of fat. The studies of Zebe (1954) on the R.Q. of butterflies during rest and during flight also indicate the utilization of fat, especially during flight. It may therefore be concluded that the higher concentration of lipase in the flight muscles of some of the butterflies we have studied is an indication of fat utilization in them. Further evidence for this view is provided by the fact that an appreciable difference in the concentration could be observed only in the case of the enzyme lipase. The failure to demonstrate lipase in the muscles of the papilionid butterflies may be due to an extremely small amount of the enzyme being present which might have been completely destroyed in the process of fixing the sections in formalin.

Another important observation is that the fibres in the muscles of the same butterfly are more or less uniform with regard to their structural and chemical

organization. Just as there is no difference in the fibre diameter, so also there is no difference in the enzymic make-up of the fibres. This was found to be the case in all the butterflies examined. The significance of this is realized more when it is taken into account that in most vertebrate skeletal muscles hitherto studied, in spite of apparent resemblances between individual fibres in a particular muscle, there are deep-seated physiological differences which become recognizable only on histochemical examination of the various enzymes contained in them (George & Scaria, 1956; George, Nair & Scaria, 1958; George, Susheela & Scaria, 1958b). In none of the insect muscles which Tiegs studied could there be seen any appreciable difference in structure among the individual fibres. The absence of clear individual variations in the structure and physiology of the fibres within a muscle, though there be such differences between different muscles in the same animal, may be regarded as major feature in which the insect muscle differs from the vertebrate skeletal muscle. This organizational difference may be directly associated with the tracheal mode of respiration which is so characteristic of insects.

SUMMARY

1. Fibre diameter and enzyme content were studied in the flight muscles of butterflies exhibiting various capacities for flight.
2. The flight muscles of the better fliers are composed of narrow fibres, while those of the poor fliers are composed of larger fibres. The fibres in any one muscle are uniform with regard to the size and the content of a few enzymes studied, viz. lipase, acid and alkaline phosphatases, ATPase and succinic dehydrogenase.
3. Quantitative estimation of the lipase activity in the flight muscles of different butterflies showed a remarkable relationship with the insect's ability to fly and the concentration of lipase in its flight muscles. Good fliers are equipped with larger quantities of lipase than are poor fliers.
4. It is suggested that, like birds and locusts, butterflies also utilize fat for energy during sustained flight.

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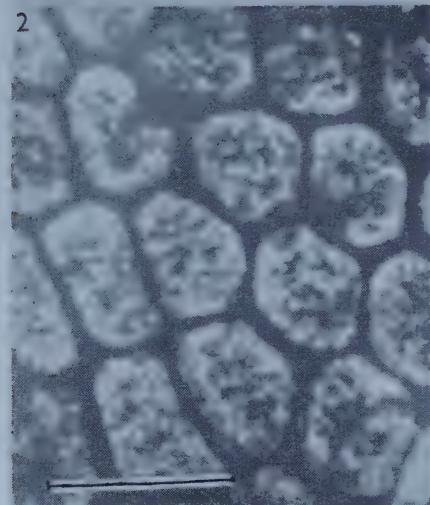
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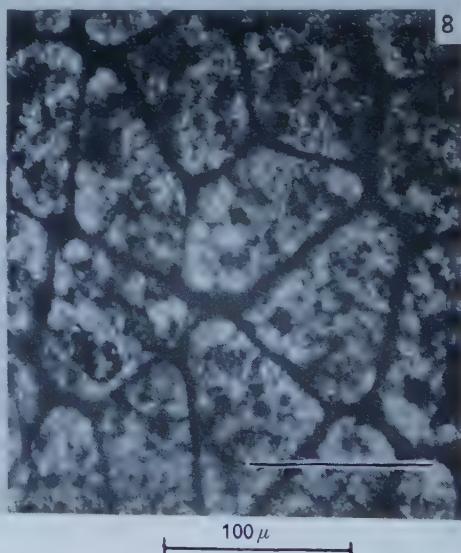
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100 μ

GEORGE AND BHAKTHAN—A STUDY ON THE FIBRE DIAMETER AND CERTAIN
ENZYME CONCENTRATIONS IN THE FLIGHT MUSCLES OF SOME
BUTTERFLIES

(*Facing p. 314*)



GEORGE AND BHAKTHAN—A STUDY ON THE FIBRE DIAMETER AND CERTAIN ENZYME CONCENTRATIONS IN THE FLIGHT MUSCLES OF SOME BUTTERFLIES

EXPLANATION OF PLATES 4 AND 5

Photomicrographs of transverse sections of the flight muscle of butterflies, lipase activity demonstrated histochemically.

Fig. 1. *Danais chrysippus*. Note the abundance of the precipitate (maximum lipase activity).
Fig. 2. *Euploea core*.
Fig. 3. *Danais limniace*.
Fig. 4. *Delias eucaris*.
Fig. 5. *Zetidus agamemnon*. Note the large size of the fibres and very little precipitate present (minimum lipase activity).
Fig. 6. *Papilio polytes*.
Fig. 7. *Precise lemonias*.
Fig. 8. *Hypolimnas bolina*.

THE THICKNESS OF SOME INSECT EPICUTICULAR WAX LAYERS

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INTRODUCTION

It is now generally accepted, following the work done principally by Ramsay (1935), Alexander, Kitchener & Briscoe (1944*a*, *b*), Wigglesworth (1945, 1947, 1948), Kramer & Wigglesworth (1950) and Beament (1945, 1955, 1958, 1959) that the impermeability of an insect's cuticle to water is due to a thin superficial layer of wax in the epicuticle.

Beament (1945), as part of an extensive investigation of insect waxes, measured the thickness of this layer on the exuvia and puparia of a variety of insects by assuming the wax to form a complete and uniformly thick layer over the surface of the cuticle and so relating the volume of wax to the area of cuticle over which it was spread. This he did by extracting the cuticle with chloroform, weighing the extracted wax and relating this quantity, expressed as a volume, to the total surface area of the sample. The inherent limitations of this method will be discussed later, but Beament found the wax layers to be from 0·2 to 0·3 μ thick.

Beament measured cuticular areas in three ways, namely by (*a*) camera lucida, (*b*) use of the formula $S = KW^{\frac{2}{3}}$ and (*c*) 'geometrical considerations'. These methods, however, will only record the apparent surface area of the cuticle, whilst the highly irregular nature of the epicuticle itself will be largely ignored. These irregularities in the surface (see Holdgate & Seal, 1956; Locke, 1959), which usually take the form of hairs and folds, although on a microscopic scale, will increase the area many times. Indeed, Glynne Jones (1955), who investigated the cuticular surface of the worker honey-bee, estimated the true area of the epicuticle of this insect to be at least ten times greater than the measured apparent area, the discrepancy being due to the additional area of the hairs and microfolds of the epicuticle.

The accurate measurement of surface area is, generally speaking, very difficult, but a technique has been developed by surface chemists which exploits the phenomenon of gaseous adsorption by solids and which enables one to measure the surface area of certain inorganic materials with some confidence. Up to the present time, however, this technique has not been tried to any great extent on biological materials. It was therefore considered that a re-examination of the question of wax thicknesses, using Beament's method in principle, but measuring the true surface area of the cuticle by gaseous adsorption, would provide an opportunity not only for testing

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the suitability of the technique for biological materials, but for enabling one to determine to what extent a true measure of the epicuticular surface alters the final value of the wax thickness.

THE MEASUREMENT OF SURFACE AREA BY GASEOUS ADSORPTION

The gas-adsorption technique for measuring the surface area of a solid makes use of the fact that when an evacuated solid comes into contact with a gas, some of the gas molecules become physically adsorbed on the surface of the solid so as to form a complete monolayer at low gas pressures and many layers at high gas pressures. Thus, provided one can determine that point at which the monolayer is complete (the monolayer capacity of the solid), calculation of the surface area is a question of measuring the total volume of gas adsorbed by the solid at that point and expressing this quantity in terms of area by calculating the number of tightly packed molecules in the adsorbed film and assuming the area occupied by the gas molecule to be equivalent to its cross-sectional area.

This is of course expressing the principles of the gas-adsorption technique in very simple terms, and for a complete account Brunauer (1945) and Gregg (1951) are recommended.

In practice one usually measures the volumes of gas taken up by a known weight of outgassed solid (maintained at a constant temperature) at increasing gas pressures and plots the volumes of gas adsorbed per gramme of solid against the equilibrium gas pressures. A plot of this kind is referred to as an adsorption isotherm and in all five types are recognized (Brunauer, Emmett & Teller, 1938; Brunauer, Deming, Deming & Teller, 1940).

The theory of multimolecular adsorption of Brunauer *et al.* (1938), later modified by Brunauer *et al.* (1940) allows one to determine from the adsorption data the monolayer capacity of a solid and hence its specific surface. The specific surface of a solid is defined as the surface area per gramme of material.

The Brunauer, Emmett and Teller (BET) equation is usually expressed in the form

$$\frac{P}{V(P_0 - P)} = \frac{1}{X_m C} + \left[\frac{C-1}{X_m C} \frac{P}{P_0} \right], \quad (1)$$

where V = volume of gas adsorbed in ml. at equilibrium gas pressure P ;

P_0 = saturated vapour pressure of the adsorbate;

X_m = monolayer capacity of the solid;

C = an energy constant.

Thus, a plot of $P/[V(P_0 - P)]$ against P/P_0 will give a straight line, of slope $(C-1)/X_m C$ and of intercept $1/(X_m C)$, from which the monolayer capacity may be calculated. The monolayer capacity of a solid, which by definition is that quantity of gas which 1 g. of solid has adsorbed at the completion of the monolayer, is related to the specific surface by the equation:

$$S = \frac{X_m N}{M} A_m, \quad (2)$$

where S = specific surface of the solid;

X_m = monolayer capacity of the solid;

N = Avogadro's number;

M = molecular weight of the gas;

A_m = area occupied by the gas molecules in the completed monolayer (Livingstone, 1949; Gregg, 1951, p. 96).

The BET theory is imperfect in many ways (Gregg & Jacobs, 1948). One major limitation is that it can only be applied with confidence to adsorption data within the relative vapour-pressure range of $P/P_0 = 0.05-0.35$. In spite of this and other limitations, the theory does provide a practical method of determining, with ease and repeatable accuracy, the specific surface of a solid from its adsorption data.

THE MEASUREMENT OF EPICUTICULAR SURFACES BY GASEOUS ADSORPTION

The main problems in applying the gas-adsorption technique to measure the surface area of insect cuticle can best be summed up in three questions. First, what is a suitable source of insect cuticle; secondly, can insect cuticle be successfully outgassed at room temperature; and thirdly, which gas should be used?

Material

Wing membranes and elytra were chosen as a suitable source of insect cuticle for the following reasons. First, both wings and elytra are isobilateral structures with an epicuticle forming the outermost layer of both surfaces. Such a structure is ideal for the present work where the gas molecules must be confined to the epicuticular surface alone. Secondly, in this method of determining the average thickness of the wax layer, one must assume that the wax forms a complete and uniformly thick layer over the surface of the epicuticle. These assumptions, which will be considered later, were thought more likely to be true of small and discrete areas of cuticle, such as wings, rather than exuvia and puparia with their heterogeneous structure; though the use of whole wings as a source of insect cuticle, as opposed to exuvia, suffers from the disadvantage that one can never be certain that the extracted wax was necessarily present in the cuticle alone.

Cuticle samples were prepared in the following way. The insects were killed with hydrogen sulphide gas and undamaged wings were removed from the thorax by pulling at their bases with a fine pair of forceps. The wings were washed in distilled water, dried between sheets of filter-paper and finally transferred to a weighed sample bulb. The tegmina of *Periplaneta americana* were not washed because of the mobile nature of the lipoid (Ramsay, 1935; Beament, 1955, 1958).

The outgassing of insect cuticle

Before the surface area of a substance can be measured by gas adsorption, the surfaces must be cleared of contaminating adsorbed molecules. This procedure is termed 'outgassing' and with inorganic materials is usually accomplished by

subjecting them to a vacuum greater than 10^{-4} mm. Hg at about 100°C . for an hour or more. It was clearly undesirable that insect cuticle should be heated to such temperatures. But outgassing is an endothermic process favoured by high temperatures, and it was therefore quite likely that if carried out at room temperatures the surfaces would not be sufficiently cleared. This problem was investigated by leaving evacuated samples of cuticle under vacuum overnight attached to the adsorption apparatus and testing the vacuum the following morning. If complete outgassing is not achieved, the gas pressure in the apparatus rises owing to adsorbed molecules leaving the sample. As the result of a series of such tests, in which different samples of cuticle were subjected to progressively longer periods of evacuation, adequate outgassing at room temperature was found to be possible provided the cuticle was subjected continuously to a vacuum greater than 10^{-4} mm. Hg for a minimum period of 100 hr.

Krypton adsorption

In the volumetric method of determining areas, measured volumes of gas of known pressure and temperature are allowed to expand into a bulb of known volume containing the evacuated sample. The resultant reductions in gas pressures are recorded and the gas laws applied to determine the volume of gas adsorbed by the sample at each addition of gas. It is usual for the sample to be kept at a constant temperature throughout the experiment, and as physical adsorption is an exothermic process favoured by low temperatures, the bulb containing the sample is usually immersed in either liquid nitrogen (b.p. 77.6°K) or liquid oxygen (b.p. 90.5°K).

The gas most frequently used in surface measurements is nitrogen and this was tried in some exploratory experiments using a slightly modified version of Tomkins & Young's apparatus (1953). But the results from these experiments were not entirely satisfactory, as the relatively low area-weight ratio of insect cuticle combined with the high saturated vapour pressure of nitrogen frequently led to the volume correction for unadsorbed gas exceeding the volume of adsorbed gas, indicating that the areas being measured were below the range of the nitrogen method. Now Beebe, Beckwith & Honig (1945) recognized the limitations in using nitrogen for measuring small surface areas (less than $1\text{ m}^2/\text{g}$), and suggested that a gas such as krypton would be more suitable because of its low saturated vapour pressure. (Viz. $1.753\text{ mm. Hg at }77.6^{\circ}\text{K.}$ (Kingston & Holmes, 1953) compared with $760\text{ mm. Hg for nitrogen.}$)

The krypton-adsorption method was accordingly investigated and an apparatus was built designed by Dr S. J. Gregg of the University of Exeter. The method proved to be satisfactory and was used for all subsequent determinations.

Areas determined by krypton adsorption usually agree to within $\pm 5\%$ of the known areas of standard materials, and in the present work agreement between individual surface measurements of a particular sample of cuticle was found to be within 2% .

APPARATUS AND EXPERIMENTAL TECHNIQUE

The krypton-adsorption apparatus

The apparatus (Fig. 1), which is built of 'Pyrex' glass, is enclosed in a constant temperature cabinet (not shown in the diagram) and is composed of the following parts:

(1) *The krypton gas system*, consisting of a cylinder of krypton gas (99-100% pure krypton, remainder xenon) and a small side-arm *F*, terminating in bulb *D*, in which the krypton may be frozen. This reduces the gas pressure in the cylinder

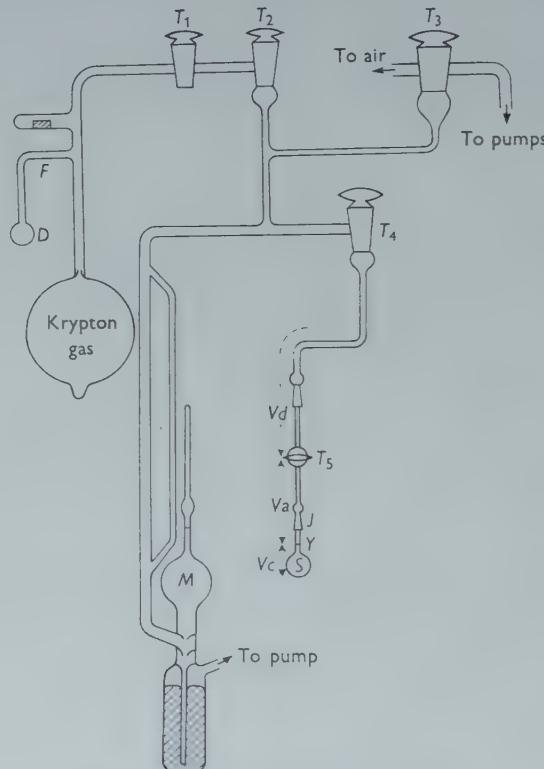


Fig. 1. Krypton-adsorption apparatus.

to just under 2 mm. Hg and facilitates a more delicate control of the quantities of gas entering the doser. The gas system communicates, via high-vacuum taps *T*₁ and *T*₂, with:

(2) *The doser* (*Vd*), calibrated for volume with krypton and consisting of that part of the apparatus delimited by high-vacuum taps *T*₂, *T*₃ and *T*₅, including an accurately calibrated Macleod gauge (*M*), designed to measure gas pressures ranging from 5×10^{-6} to 7.0 mm. Hg.

(3) *The dead-space system*, consisting of *Va*, the dead-space volume above the liquid nitrogen level *Y*, and *Vc*, that volume of the sample bulb immersed in liquid nitrogen. Both volumes were calibrated by weighing with mercury.

Before the volume of the doser can be calibrated, or indeed any determinations made, the apparatus has to be outgassed in order to free the glass of adsorbates. This was done by evacuating the apparatus and flaming the glass with a gas-air hand-torch to a temperature of about 200° C., this operation being repeated until the apparatus was capable of maintaining a vacuum greater than 10^{-4} mm. Hg, when left overnight with high-vacuum tap T_3 shut.

Experimental technique

A bulb containing a weighed sample of cuticle is attached to the apparatus at ground-glass joint J , and the whole apparatus evacuated via tap T_3 , by means of a mercury distillation pump backed by a single-stage rotary pump, so that a vacuum greater than 10^{-4} mm. Hg is maintained. The cuticle is outgassed for 100 hr. Bulb D and the sample bulb S are immersed in liquid nitrogen, the latter up to calibration mark Y , where it is maintained throughout the experiment. Taps T_3 and T_5 are closed and a dose of krypton is let into the doser; the pressure is measured on the Macleod gauge, the mercury levels being read with a cathetometer. T_1 , the temperature of the cabinet, is recorded and tap T_5 is opened. A period of 15 min. (previously determined) is allowed for equilibrium to become established and the equilibrium pressure P_2 and the cabinet temperature T_2 are recorded.

This procedure is repeated with successive doses of gas and may be continued until the saturated vapour pressure of krypton is reached, if a complete isotherm is required, or simply limited to the BET region of the isotherm (viz. $P/P_0 = 0.05 - 0.35$) if a measure of the surface alone is required.

By application of the gas laws the volumes of gas adsorbed by the sample (V_{ad} , ml.) may be calculated from the equation

$$V_{\text{ad.}} = \left[\frac{P_1 T_a}{T_1 P_a} V_d - \frac{P_2 T_a}{T_2 P_a} V_d \right] - \frac{P_2 T_a}{P_a} \left[\frac{V_d + V_c}{T_2} + \frac{V_c'}{T_c} \right],$$

where

P_1 and T_1 are respectively the pressure and temperature of the doses of gas;
 P_2 and T_2 are respectively the pressure and temperature of the gas at equilibrium;

V_d and V_a are respectively the volume of the doser and the dead-space above the liquid nitrogen level;

$V_c' = (V_c - V_s)$, where V_c is the total volume of the sample bulb below the liquid nitrogen level and V_s is the volume of the cuticle sample (obtained by relating the weight of cuticle to its density; assumed to be 0.96 g./c.c.).

P_a and T_a are respectively normal temperature and pressure (N.T.P.).

T_c is the temperature of the liquid nitrogen bath.

Data so obtained were plotted first as an adsorption isotherm and then as a BET plot. The monolayer capacity of the sample was then calculated by means of the BET equation (equation 1). Before the specific surface of the sample can be calculated from the monolayer capacity (equation 2), the cross-sectional area of

the krypton molecule must be known. Although several different values are given in the literature, depending upon which property of the molecule has been used in the determination (Davis, De Witt & Emmett, 1947; Livingstone, 1949; Davis, Shuler & Weaver, 1950; Kingston & Holmes, 1953), that of 19·5 sq. Å., suggested by Beebe *et al.* (1945) has been used throughout the present work.

RESULTS AND ANALYSIS OF THE BET AREAS

Some typical krypton adsorption isotherms of insect wings and elytra, together with the corresponding *BET* plots of the adsorption data, are given in Fig. 2. In all cases, the adsorption isotherms were found to conform to type 2 of the *BET* classification (Brunauer *et al.* 1940). This type of isotherm, which indicates the absence of very narrow pores (< 10 Å. in diameter) in the surface, occurs frequently in surface chemical work and is one of the more fully understood isotherms to which the *BET* procedure can be applied with some confidence.

The krypton adsorption areas (hereafter referred to as the *BET* areas) of those wings and elytra which were investigated are given in Table 1, column 4. A comparison of these values with the corresponding apparent areas (Table 1, column 8*b*) gives a ratio of 1·6 for the wing membranes and 6·7, 7·3 and 8·2 respectively for the elytra, with an average ratio of 4·1. In the present work, the apparent area of a particular wing was determined by projecting the outlines of 10 of them on to a graduated screen and measuring the area covered by the enlarged images. This projection method was considered to be at least comparable to the three methods used by Beament (1945). In addition, microscopical examinations of the wing surfaces were made in an attempt to account for the area of the hairs which frequently occur in large numbers on the surface and which were likely to provide an important correction to the final apparent area. It is possible to calculate the total apparent area of these hairs by measuring their size and density and assuming their shape to be that of a cone. The relative importance of this additional area can be judged by comparing the values given in Table 1, columns 8*a* and 8*b*, where to take two extreme examples, it may range from as little as 6% of the apparent area, as in the case of the forewings of the worker honey-bee, *Apis mellifera*, to as much as 50%, as in the case of the wings of the tsetse fly, *Glossina morsitans*.

It is of some interest to note that the experimentally determined *BET* areas of the wing membranes (Table 1, column 4) are in the same relative order as the corresponding apparent areas (Table 1, column 8), and that the ratio of *BET* area to apparent area varies from one insect to another and from one part of the body to another.

On microscopical examination, the surfaces of the elytra, although showing considerable ridging, were found to be free of hairs and the relevant values in Table 1, column 8*c*, are the projected areas only.

The main problem in interpreting the *BET* areas is to decide whether the gas molecules have been confined to the outer surface of the epicuticle, for that is where

the wax layer is situated, or whether the gas has penetrated into the substance of the wing, so recording the general internal area as well as the outer, superficial area. Although no direct evidence can be cited on this question, we can deduce indirectly from the data that the gas is confined to the outer epicuticular surface. Perhaps the most striking single piece of evidence is provided by the areas of those wings and elytra which were extracted with petroleum ether. In Table 1, column 4, the BET areas of extracted wings of *G. morsitans* and the extracted elytra of

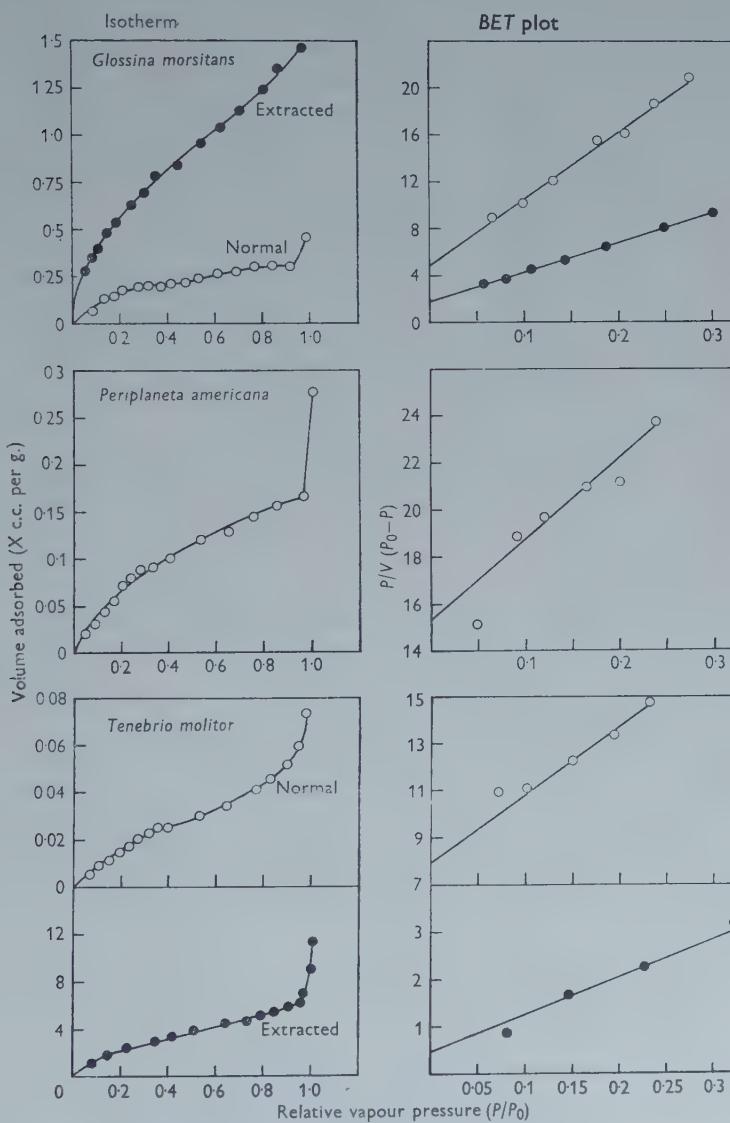


Fig. 2. The krypton adsorption isotherms of insect cuticle and the BET plots of the adsorption data.

Table I. The BET and apparent area of insect wings and the average thickness of the epicuticular wax layer

Materials and species (1)	Total number of wings in sample (2)	Total BET area of sample (cm. ²) (3)	Average BET area of wings (cm. ²) (4)	Total weight of wax extracted from sample (g.) (5)	Average thickness of wax layer* (μ) (6)	Average apparent area of wing (cm. ²)		Corrected for hairs (8b)	Total apparent area of sample (cm. ²) (9)	Average thickness of wax layer* (μ) (10)	Number of molecular layers† (11)
						Number of molecular layers† (7)	Uncorrected (8a)				
(1) Wing membrane											
(a) <i>Musca</i> <i>domestica</i>	1150	531.86	0.46	0.0056	0.11	/	0.20	0.28	322.00	0.18	18
(b) <i>Protophormia</i> <i>terrestris-novae</i>	1068	742.82	0.70	0.0082	0.12	1.2	0.32	0.45	480.60	0.18	18
(c) <i>Glossina</i> <i>moseriensis</i>	950	837.19	0.88	0.0101	0.12	1.2	0.34	0.51	484.50	0.22	22
(i) Extracted	950	1946.66	2.05	—	—	—	—	—	—	—	—
(d) <i>Apis mellifera</i> (forewings)	1681	1033.22	0.62	0.0129	0.13	1.3	0.34	0.38	638.78	0.21	21
(2) Elytra											
(a) <i>Periplaneta</i> <i>americana</i>	24	1026.15	42.76	0.0104	0.11	1.1	5.20	124.80	0.87	87	
(b) <i>Dysdercus</i> <i>fasciatus</i>	219	954.62	4.36	0.0365	0.40	4.0	0.60	131.40	2.89	289	
(c) <i>Tenebrio</i> <i>molitor</i>	502	1421.09	2.83	0.1713	1.26	1.26	0.42	210.84	8.46	846	
(i) Extracted	270	6219.57	23.04	—	—	—	—	—	—	—	—

* Relative density of wax assumed to be 0.96 g./c.c. † Length of wax molecule assumed to be 100 Å.

Tenebrio molitro are given. Comparing these areas with the corresponding values for the untreated wings, we can see that as the result of extraction, the area is more than doubled in the case of *Glossina* and increased more than tenfold in the case of *Tenebrio*. It is difficult to explain these differences in area solely in terms of an increase in the external surface of the epicuticle. Removal of the superficial wax layer will almost certainly open up the pore canal system of the cuticle to the krypton molecules, which in turn will probably enable the gas to reach the exocuticle and endocuticle. It is much more likely that with an extracted wing, krypton is penetrating into the substance of the cuticle, being adsorbed on to all available internal surfaces and so recording the internal area as well as the external, superficial area. This does not appear to happen with a normal wing. Furthermore, if krypton is penetrating into the cuticle it will only do so by diffusion, which at liquid nitrogen temperatures will be very slow. This point was carefully checked early in the work by determining experimentally the times taken by the krypton-cuticle system to reach equilibrium at increasing gas pressures. In all cases, equilibrium was found to be reached quickly and within 15 min. Finally, supporting evidence is provided from some experiments in which the BET area of a sample of normal wings was determined, followed by a similar determination in which the wings were cut into small pieces. The average area of the cut wings was always considerably greater than that of normal wings. In conclusion, one is struck by what we may term the 'reasonableness' of the BET areas when compared with the corresponding apparent areas. The greatest difference that we find between the two is just under tenfold, in the case of the tegmina of *Periplaneta americana*, and in the majority of cases the difference is much lower than this. If the BET area included both the superficial and the general internal area, one would expect the difference to be much greater.

So far we have only examined the possible errors in the area measurements when considering the wing as a whole structure, but clearly the physical condition of the outer layers of the cuticle will also affect the surface area as measured by gas adsorption. Holdgate & Seal (1956) demonstrated the presence of a 'bloom' of wax over the surface of the pupal cuticle of *Tenebrio molitor* and such a layer if present generally throughout the cuticle of insects will greatly increase the area. The presence of a 'bloom' of wax may be one explanation for the relatively large areas of the elytra, though the low area ratio of the wing membranes suggests that such a layer is unlikely to be present in this case. Due consideration must also be given to the effects which the rather rigorous experimental conditions may have on the cuticle. The prolonged period of outgassing at room temperature is likely to cause distortion and shrinkage of the cuticle and possibly the formation of fissures in the surface layers. In addition, high vacuum is likely to remove solvent from the wax so producing a more porous layer; this latter effect may well be operating in the case of the grease of *Periplaneta americana* (Beament, 1955). Considering the wing membranes first, once again the low area ratio of 1·6 does suggest that the effects on the surface are comparatively slight, though with the elytra they may be a factor contributing to the high values.

Another possible source of error in the *BET* method which must be considered is the possibility of krypton entering the vein system of the wings via the torn wing bases. This point was checked by measuring the area of a sample of wings in which the base of each wing was sealed with high-vacuum 'Apiezon' wax. The results of this experiment indicated no appreciable difference in area between normal and sealed wings. This is certainly difficult to explain, but it may be due to the blood in the veins blocking the broken ends on clotting.

Dennell (1958) showed that atmospheric oxygen was unable to penetrate through the larval epicuticle of *Calliphora vomitoria*, and in the present work all the evidence does seem to indicate that the krypton is being confined to the outer surface of the epicuticle by the wax layer. As a result we may conclude that the *BET* method gives a reasonably accurate measure of the true surface area of the epicuticle.

THE EXTRACTION AND CALCULATION OF THE AVERAGE THICKNESS OF THE WAX LAYER

After the *BET* area of a sample of wings was determined the wax was removed by refluxing with two successive lots of 50 ml. of petroleum ether (b.p. 60–80° C.) for 15 min. at a time. The solution was filtered through filter-paper washed in hot petroleum ether and the wings were rinsed several times with fresh solvent to remove any solution remaining on the surface. The wax solution was concentrated, transferred to a weighing bottle and allowed to evaporate to dryness. The resulting wax was then weighed and the average thickness of the wax layer calculated by dividing the volume of wax (relative density assumed to be 0·96 g./c.c.) by the total *BET* area of the sample.

The average thickness of the wax layers of those wings and elytra which were investigated are given in Table 1, column 6, whilst in column 10, for comparison, the thicknesses of the same layers are given, calculated from the total apparent areas.

DISCUSSION

We can see from Table 1, column 6, that the wax layers of the wing membranes are remarkably similar in thickness, varying from 0·11 to 0·13 μ . Those of the elytra, however, differ considerably in thickness and show a progressive increase from *Periplaneta americana* (0·11 μ) to *Tenebrio molitor* (1·26 μ). This variation may be explained if we remember that elytra are essentially modified forewings in which the original function of flight has been superseded, to a varying degree by that of protection. Now the three insects *Periplaneta*, *Dysdercus* and *Tenebrio* provide a series in which the degree of modification of the forewings progressively increases and we can see that as the degree of modification increases so does the thickness of the epicuticular wax layer. In the cockroach, *Periplaneta*, the forewings are only slightly modified and the wax layer is similar in thickness to that of an unmodified wing membrane (0·11 μ). The hemelytra of the adult cotton-stainer, *Dysdercus*, provide an intermediary condition in which the anterior half of the wing is elytrum and the posterior half is membrane. Here the average wax thickness is 0·4 μ .

Finally, in the adult meal-worm, *Tenebrio*, true elytra are present, the principal function of which is the protection of the hindwings and the excessively thin dorsal tergites. In these true elytra the wax thickness is 1.26μ . The presence of such a thick layer is not difficult to explain, as Wigglesworth (1948) found that there is a continuous secretion of wax throughout the adult life of *Tenebrio*. This lipid on examination was found to be a soft wax which could easily be removed from the surface of the cuticle, and it may well be that, as *Tenebrio* is a burrowing insect in which the elytra provide the main protection of the body, a continual supply of wax is necessary to replace that which is being continually removed by abrasion. Also, a thick layer of soft wax such as this has the advantage of providing an immediately available reserve, which can spread rapidly over the abraded areas of cuticle, so completing the all-important monolayer, before freshly secreted wax reaches the area.

In this method of measuring the thickness of the epicuticular wax layer we must assume first that the wax forms a complete layer over the surface of the cuticle and secondly that the wax is uniform in thickness. We can infer from the BET areas that the epicuticular wax forms a complete covering to the surface of the wings and indeed it is difficult to see how the wax layer can function as efficiently as it does if it is discontinuous. The first of these assumptions then may be taken as being reasonably correct. In the case of the second assumption, however, Shaw (1955) found that the wax layers of the young stages of *Locusta* and *Sialis lutaria* varied in thickness in different parts of the body cuticle. Unfortunately, Shaw does not describe how he measured cuticular areas so that a precise comparison of his results with those of the present work is not possible, although it should be noted that the variations in thickness which he found could equally be due to a variation in the ratio of true area to apparent area at the different parts of the body. As the present work has shown (Table 1, columns 4 and 8) this ratio varies from one insect to another and from one part of the body to another. We can only conclude that the wax layer may vary in thickness throughout the body cuticle but, as was mentioned earlier, wing membranes and elytra were used as sources of insect cuticle in an attempt to minimize this possible inaccuracy.

The average value of 0.25μ suggested by Beament (1945) for the thickness of an insect's wax layer can be seen to be more than double that obtained in the present work for wing membranes, and from this point of view it would be more correct to speak of an average thickness of 0.12μ . This difference in the two average values is probably due almost entirely to a more accurate measurement of surface area by the gas adsorption method. For comparison the wax thicknesses calculated from the total apparent areas of the cuticle samples have also been included in Table 1, column 10, and these values are also less than Beament's average figure, although if the uncorrected apparent areas are used in this calculation (Table 1, column 8a), the values average out to 0.25μ . Mention has already been made of the variation in thickness of the elytral wax layers and clearly it is meaningless to speak of an average thickness in this case. Indeed, this argument may well be extended to the wax layers of insects in general. The number of molecular layers represented by

the various wax layers are given in Table 1, columns 7 and 11, and have been calculated by assuming the wax molecules to be vertically orientated throughout the layer and their chain-length to be 100 Å. (Beament, 1945).

In conclusion, brief mention may be made of more recent work by Beament (1958). By accurately measuring the loss of water from large nymphs of the cockroach, *Periplaneta americana*, and taking the wax thickness as 0.25μ and the total surface area to be 7.5 cm^2 , Beament was able to calculate the absolute water permeability of the cuticle. It is of some interest to repeat this calculation, substituting the appropriate values for wax thickness and surface area obtained in the present work, for those used by Beament. If we accept the wax thickness to be 0.11μ and assume the total true area of the nymph to be eight times greater than Beament's figure for the total apparent area (Table 1) then the absolute permeability of the cuticle of the cockroach comes to 0.13×10^{-8} absolute units compared with 1.65×10^{-8} absolute units given by Beament. This extremely low water permeability reflects the highly organized nature of the epicuticular wax and emphasizes its efficiency as a water-proofing layer.

SUMMARY

1. The average thickness of the epicuticular wax layers on the wing membranes and elytra of a number of different insects has been measured by relating the volume of extracted wax to the area of cuticle over which it was spread.
2. The true surface area of the epicuticle was measured by krypton adsorption.
3. The ratio of absorption area to apparent projected area was found to be 1.6 for the wing membranes, and 6.7–8.2 for the elytra, with an average value of 4.1.
4. The wax layers were found to be remarkably similar in thickness on the wing membranes, ranging from 0.11 to 0.13μ but to vary from 0.11 to 1.26μ in the case of elytra, where the wax thickness appears to be related to extent of modification.

The work was done during the tenure of a research studentship awarded by the Agricultural Research Council and forms a part of a thesis for the degree of Ph.D. of London University. I wish to thank Dr A. B. P. Page, Imperial College, London, for his help and encouragement throughout the work and Dr S. J. Gregg of Exeter University, for his instruction in the principles of surface chemistry.

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GLIDING FLIGHT OF THE FULMAR PETREL

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INTRODUCTION

The fulmar petrel (*Fulmarus glacialis*) is one of the very few species of British breeding birds which can be watched in flight at close range, under natural conditions and without causing it any alarm. A qualitative account of its manner of flight and the control movements which it uses has been given by Pennycuick & Webbe (1959). The present paper presents a quantitative account of its performance in gliding flight.

THEORY

The conclusions presented below are based on measurements of the lift and drag coefficients (C_L and C_D) developed by fulmars in gliding flight. These were obtained from the usual formulae:

$$C_L = \frac{2L}{\rho V^2 S}, \quad (1)$$

$$C_D = \frac{2D}{\rho V^2 S}, \quad (2)$$

where L is the lift, D the drag, ρ the air density, V the airspeed and S the wing area. The measurements of lift and drag are based on the following assumptions.

In a bird gliding at an angle α below the horizontal with wings level, the lift and drag are given by

$$L = W \cos \alpha + Ma_n, \quad (3)$$

$$D = W \sin \alpha - Ma_l, \quad (4)$$

where W is the weight, M the mass, and a_n , a_l are the accelerations normal to and in line with the direction of motion respectively. In practice the method of tracking did not lend itself to measuring the accelerations with the necessary accuracy, and so they were ignored. This is equivalent to assuming that all the birds were gliding in a straight line at constant speed, and although this is not in itself a good assumption a sufficient number of measurements was obtained to draw some reasonably solid conclusions from the resulting scatter.

The formulae for lift and drag coefficients now become

$$C_L = \frac{2W \cos \alpha}{\rho V^2 S}, \quad (5)$$

$$C_D = \frac{2W \sin \alpha}{\rho V^2 S}. \quad (6)$$

METHOD

The five quantities on the right-hand side of equations (5) and (6) were estimated as follows.

Weight

No method has been hit upon for estimating the weights of individual birds without catching them. All the birds were therefore assumed to weigh 1·6 lb., from the average given in Fisher's (1952) monograph.

Air density

A value of 0·0024 slugs per cubic foot was used throughout.

Wing area

This can be varied in flight. The outlines of Fig. 1 were obtained by holding a fulmar down on a piece of graph paper and drawing round it. Outline 1 with tail spread has about 1·9 times the area of outline 4 with tail furled. A photograph of each bird taken at the moment for which the speed was calculated, was compared with these outlines, and the prevailing wing area estimated therefrom.

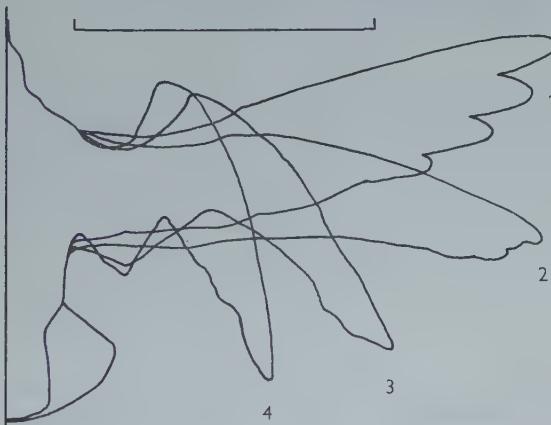


Fig. 1. Variation of wing area in different positions. Areas exclusive of tail: 1, 1·3 sq.ft.; 2, 1·1 sq.ft.; 3, 0·9 sq.ft.; 4, 0·8 sq.ft. Area of tail: furled 0·1 sq.ft., spread 0·2 sq.ft. Scale: 1 foot.

Airspeed and gliding angle

These were obtained by tracking the birds optically from a point on the cliff top. The tracking device was based on a camera mounted on a pan-tilt head, the azimuth and elevation movements of which were connected by Bowden cables to two pens writing in different colours on a moving paper chart. Attached to the camera was a rangefinder of 8 in. base whose ranging knob was connected by another Bowden cable to a third pen on the recorder.

The recorder was started when an approaching bird reached a range of about 65 ft., and the device was then kept trained on the bird with the rangefinder following

its approach: the pen recorder thus produced a record of the bird's azimuth, elevation and range from the camera position, in other words a plot of its position in polar co-ordinates as a function of time.

When the bird reached a distance of 35 ft., at which the camera was focused, an arm projecting from the ranging knob pressed a microswitch which tripped an electric shutter release and also operated a relay which marked the chart. The end product was thus a photograph of the bird for which its position was known in polar co-ordinates, and also the rates of change of these co-ordinates. From this information its vector groundspeed was calculated in rectilinear co-ordinates.

The vector windspeed was obtained from a whirling cup anemometer fitted with fins and mounted in gimbals on the end of a pole, so that it orientated itself into wind both horizontally and vertically. It was held in the region where the fulmars were flying, and the windspeed and the angles which the anemometer took up were noted, the operation being carried out before (or in the middle of) a series of observations, and the wind assumed constant. The information was converted into the three rectilinear components of windspeed, referred to the same axes as those of the bird's groundspeed. The three components of airspeed were then obtained by subtracting the windspeed from the groundspeed, and hence the scalar airspeed and the angle of glide were calculated.

Accuracy

The accuracy of the groundspeed measurements is thought to be about $\pm 10\%$. To this must be added an unknown error due to variations in windspeed, since this was not continuously recorded; this error is at its worst in gusty conditions. Together with the doubt attaching to the weight and wing area, it is thought not unreasonable to claim an accuracy of $\pm 20\%$ for the calculated values of the coefficients, neglecting errors due to accelerations.

RESULTS

Range of speed

Fig. 2 shows the distribution of speed measurements grouped at intervals of 5 ft./sec. The average of the 111 measurements there represented is almost exactly 40 ft./sec., the greatest proportion (24%) falling between 35 and 40 ft./sec. Over 96% of the measurements fall between 22 and 65 ft./sec.

Range of lift coefficient

Fig. 3 shows the relation between lift coefficient and airspeed. The regularity of the curve is not surprising since the airspeed is used to calculate the lift coefficient, the scatter being mainly due to variations in wing area. The important feature of the diagram is that the main mass of measurements goes up to $C_L = 1.8$, above which there are only four scattered observations, no doubt representing birds in a stalled condition. If the maximum lift coefficient is taken to be 1.8, the stalling speed with maximum wing area would be about 23 ft./sec.

A maximum lift coefficient of 1.8 is rather high for the Reynolds number concerned (5×10^4), at which a maximum of 1.2 would be more likely according to glider practice (Welch, Welch & Irving, 1955). However, gliders are constructed for a much lower minimum drag coefficient than prevails in the fulmar. For a high lift, low speed wing, the figure of 1.8 is not unreasonable, and its achievement is doubtless facilitated by efficient slotting by the alula and splayed primaries, and a flap effect produced by spreading the tail. Lift coefficients over 2 can be obtained by these means in aircraft (Baird, 1939).

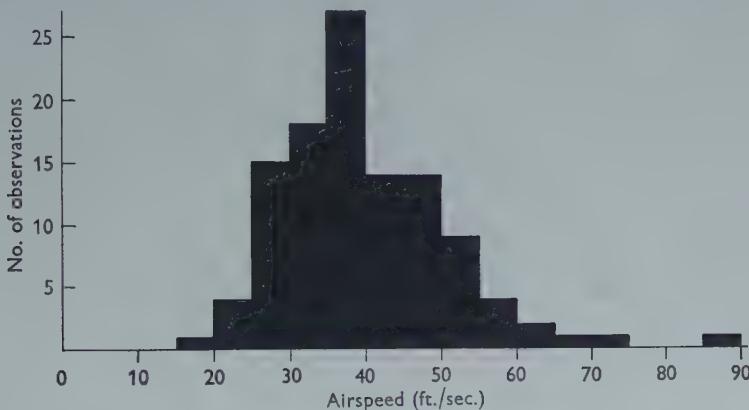


Fig. 2. Distribution of airspeed measurements in steps of 5 ft./sec.

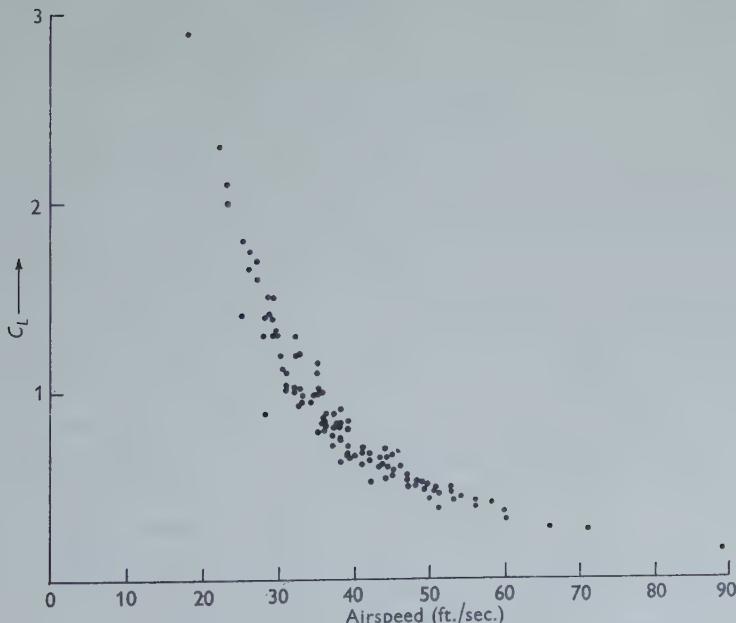


Fig. 3. Relation between lift coefficient and airspeed.

In this connexion it is of interest to note that, as in other birds (Schufeldt, 1890), the alula is supplied by a branch of the tendon of the extensor digitorum communis, so that when the wing is swept fully forward and spread for minimum speed, the alula would be extended automatically.

Variation of wing area with speed

Although there is no rigid connexion between wing area and speed, there is a significant tendency to reduce wing area with increasing speed as shown in Fig. 4, thus reducing the range of variation of lift coefficient needed to effect changes of speed.

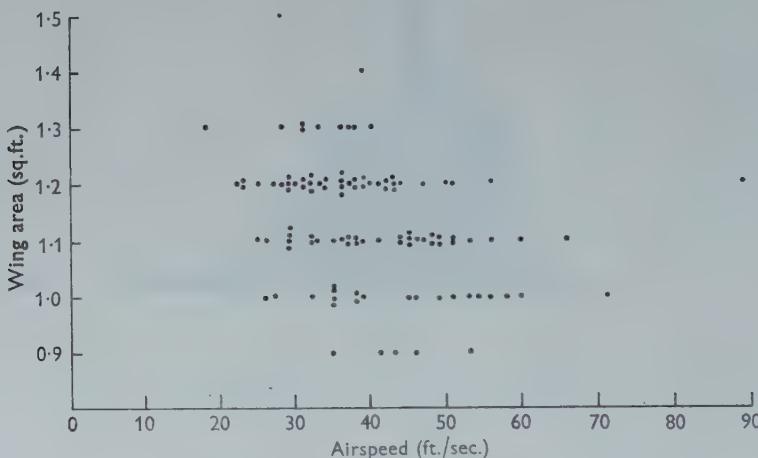


Fig. 4. Relation between wing area and airspeed. The correlation coefficient is -0.36 , and $P < 0.01$.

Connexion between drag coefficient and foot positions

When fully retracted the feet are folded forwards and concealed under the flap of flank feathers which covers the leading edge of the wings when they are folded. They are then entirely invisible (position 0). When the bird is manoeuvring near the cliff the feet are generally lowered, and if not required they are then carried close together with webs furled below the tail (position 1). From here they can be lowered, with webs spread, into the airstream. Up to 20° of lowering is position 2, more than 20° is position 3.

Fig. 5 shows the drag coefficients produced at these different foot positions. While the readings for position 0 are fairly well bunched, as soon as the feet are brought out from this position the scatter (whose causes have been considered) at once increases. This is explained by the observation that the feet are only kept fully retracted when the bird is gliding steadily along in smooth conditions, and as soon as gusty conditions are met with, or manoeuvres called for, the feet appear. Thus neglected accelerations are more likely to give rise to errors in foot positions 1-3 than in position 0.

In spite of the increased scatter, it is evident that the drag coefficient increases as soon as the feet appear, even though they be folded beneath the tail (position 1), and that lowering them produces further increases in drag. They are used, in other words, as airbrakes.

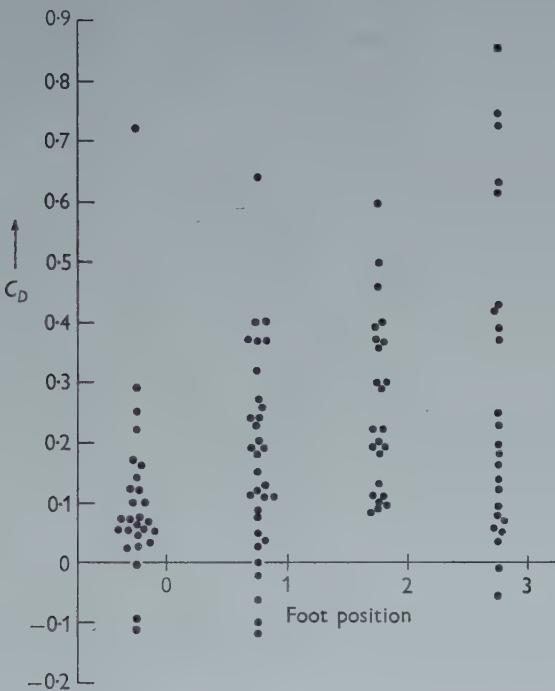


Fig. 5. Variation of drag coefficient with foot position. See text for explanation of foot positions.

Power required for level flight

Dickinson (1928) gives a value for the maximum power output for a short period by human muscle as 0.024 h.p./lb., using a bicycle ergometer. In continuous activity this is limited by the rate at which oxygen can be supplied to the muscles to 0.01 h.p./lb., if an oxygen debt is not to be incurred (Henderson & Haggard, 1925). Taking into account the more efficient ventilation to be expected in a bird's lungs (Sturkie, 1954), 0.02 h.p./lb. seems a reasonable value to take for continuous activity. The three pectoralis muscles of both sides of a fulmar together weigh 0.19 lb., and if these are assumed to provide the power used in flapping flight, there will be 0.0038 h.p. available.

From the C_D measurements for foot position 0 shown in Fig. 5 it can be seen that there is a cluster of points around $C_D = 0.06$. If this is taken as the minimum drag coefficient, then with a wing area of 1.2 sq.ft. the speed attainable would be 29 ft./sec. This is well above the stalling speed, but seems rather low for cruising, and would leave little power in hand for climbing; on the other hand, fulmars do not seem to be

capable of anything more than a very shallow climb in flapping flight. The result is of the right order of magnitude, but it should be borne in mind that the estimates of power required and of power available are both subject to some doubt.

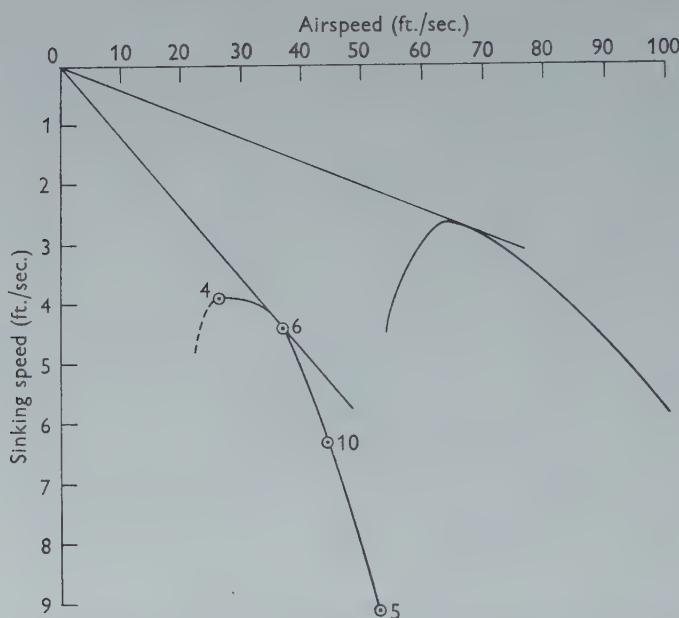


Fig. 6. Performance diagram of the gliding fulmar (left) compared with that of a typical medium performance glider (after Welch *et al.* 1955).

Performance diagram

Glider performance is often expressed in terms of a plot of sinking speed against airspeed. This curve is convex upwards and the minimum gliding angle can be found by drawing a tangent to the curve from the origin. In Fig. 6 the curve obtained for the fulmar is shown alongside that for a typical medium performance glider (after Welch *et al.* 1955). The fulmar curve has been obtained from the measurements on birds with their feet in position o, by averaging the speeds and rates of sink of all those between 20 and 29 ft./sec. to give the first point, likewise with observations between 30 and 39 ft./sec. for the second, and so on. The number of measurements averaged for each point is shown beside the point. The minimum gliding angle for the fulmar is thus about 1 in $8\frac{1}{2}$, as against 1 in 25 for the glider, and its minimum rate of sink in nearly 4 ft./sec. as against just over $2\frac{1}{2}$ ft./sec. for the glider. It should be remembered, however, that a gliding fulmar is not a glider but a powered aircraft with its motor idling, and in this context its performance is by no means contemptible.

DISCUSSION

The overall picture which emerges is that of a craft adapted to low-speed flight which has accepted some sacrifice of gliding performance. The various observations fit together fairly well, and there are no serious anomalies to be explained. There is not, for example, any need to invoke laminar flow or other subtleties to explain the results, as was found necessary by Raspé (1950) to account for his measurements on the black buzzard *Coragyps atrata*.

A question which can be considered theoretically is whether or not the fulmar is capable of dynamic soaring (Lord Rayleigh, 1883), using the wind gradient over the surface of the sea as their larger relatives the albatrosses do (Idrac, 1924a). This method of soaring has been analysed quantitatively by Walkden (1925), who showed that if a bird is to climb into wind without losing airspeed there must be a certain minimum rate of change of windspeed with height, which can be calculated from the bird's airspeed and gliding angle.

Using an airspeed of 35 ft./sec. and a gliding angle of 1 in $8\frac{1}{2}$, and assuming the wind gradient to have the form given by Idrac (1924b), Walkden's equations show that to soar over a height range of 20 ft. (selected as a reasonable minimum), the fulmar would require a windspeed of 105 ft./sec., or 62 knots, at the surface. Seamen seldom have much time to spare for bird watching under such conditions, and it may be that fulmars do soar in this way in winds of this strength. However, their normal mode of progression over the sea seems to be to climb sharply in the lift above a wave, bank steeply and dive down into the trough, flap the wings for a few strokes to reach the windward slope of the next wave, and so on.

The wandering albatross was found by Idrac to have a gliding angle of about 1 in 18 at speeds around 70 ft./sec., which allows it to soar easily in winds of 20 ft./sec. or so at the surface. The fulmar thus seems to have sacrificed the ability to soar in this way in order to be able to fly at low speeds and thus exploit the upcurrents formed on cliff faces in light winds. Indeed it is the fulmar's extraordinary skill at, and predilection for, soaring in front of cliffs which makes it such a suitable object for study.

SUMMARY

1. The basis used for estimating lift and drag coefficients is explained. A method of obtaining a photograph of a bird flying at known airspeed and rate of sink is described.

2. 96% of the speed measurements fall between 22 and 65 ft./sec., the average being 40 ft./sec.

3. A maximum lift coefficient of 1.8 can be achieved. Wing area is reduced with increasing speed.

4. The feet are used as airbrakes.

5. A comparison of the minimum drag coefficient (0.06) with the maximum estimated power output of the pectoral muscles leaves only a narrow margin of power available for climbing.

6. The performance diagram gives a minimum gliding angle of 1 in $8\frac{1}{2}$, and a minimum sinking speed of just under 4 ft./sec.

7. The fulmar has apparently sacrificed the ability to soar dynamically over the sea in order to be able to fly slowly and thus utilize light upcurrents at cliff faces.

I am very grateful to Mr Peter Davis, warden of Fair Isle Bird Observatory, Shetland, both for his personal help and for making available the facilities of the observatory, where this work was done. I am also indebted to Dr K. E. Machin for a great deal of help and advice, especially with the design and construction of the apparatus, and to Dr M. R. Head for allowing me to use a wind tunnel at the Engineering Laboratory, Cambridge, for calibrating the anemometer. Also Dr Machin and Dr R. H. J. Brown were kind enough to read the manuscript and made some valuable suggestions which have been incorporated. This work was carried out during the tenure of a D.S.I.R. Research Studentship.

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THE COMPOSITION OF THE HAEMOLYMPH OF THE LARVA OF *DROSOPHILA MELANOGASTER*

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INTRODUCTION

It has been shown by Waddington (1959) that larvae of *Drosophila melanogaster* (Oregon K) can be selected over several generations to survive and develop on a medium containing 7% NaCl. As few species are known that can survive in such concentrated media, it was considered of interest to study the haemolymph composition of the normal and selected strains. Some previous analyses of the haemolymph of normal *D. melanogaster* larvae have been given by Gloor & Chen (1950) and Zwicky (1954), but these did not include sodium or potassium estimations.

MATERIALS AND METHODS

Cultures were obtained of normal *D. melanogaster* (Oregon K) larvae and of *D. melanogaster* (Oregon K) larvae selected to survive on a medium containing 7% NaCl, hereinafter referred to as 'selected' larvae. These were grown on standard medium (agar-yeast-maize meal-treacle-water) or on standard medium containing 7% NaCl or 7% KCl.

Full-grown larvae were removed from their medium, rinsed in distilled water, dried on filter-paper and placed under liquid paraffin in a lacquered watch-glass. The body wall was punctured and haemolymph was sucked into a fine pipette. To obtain large samples the haemolymph from 10 to 40 larvae was pooled. A haemolymph sample was stored under liquid paraffin in a lacquered watch-glass. The osmotic pressure, sodium, potassium and chloride concentrations were determined as described previously (Croghan, 1958b). As far as possible a determination was carried out in duplicate or triplicate on a sample, and in many cases all the determinations were carried out on the same pooled sample.

Samples of the media were also analysed. A 1 g. sample was diluted to 5 ml. with distilled water, mixed and centrifuged. Determinations were carried out on the supernatant and the results multiplied by the dilution factor.

RESULTS

Analyses were carried out on normal larvae developing on standard medium and on 'selected' larvae developing on standard medium containing 7% NaCl. It was found very surprisingly that both normal larvae and 'selected' larvae could survive and develop on standard medium containing 7% KCl. Analyses were carried out on 'selected' larvae developing on standard medium containing 7% KCl.

The results are summarized in Tables 1 and 2. The derived results are obtained using the means of the measured results. That part of the cation concentration not accounted for by chloride is regarded as organic anion. The osmotic pressure deficit is that part of the osmotic pressure not accounted for by ionized salts, assuming that the organic anion is divalent. As calcium and magnesium were not estimated, the derived results underestimate the organic anion concentration and overestimate the osmotic pressure deficit.

Table 1. *The composition of the haemolymph of Drosophila melanogaster larvae*

Group	Osmotic pressure (mM./l. NaCl)			Organic anion (m-equiv./l.)			Osmotic pressure deficit (mM./l. NaCl)	Na/K	Cl/organic anion
	Na (m-equiv./l.)	K (m-equiv./l.)	Cl (m-equiv./l.)	equiv./l.)	NaCl)				
1	171 ± 1 (4)	52 ± 1 (5)	36 ± 1 (5)	30 ± 1 (8)	58 ± 2	98 ± 2	1.44 ± 0.04	0.57 ± 0.05	
2	199 ± 2 (7)	90 ± 4 (5)	37 ± 5 (5)	67 ± 3 (5)	60 ± 7	87 ± 6	2.43 ± 0.14	1.12 ± 0.12	
3	181 ± 4 (6)	35 ± 2 (5)	48 ± 3 (5)	71 ± 2 (3)	12 ± 4	101 ± 5	0.73 ± 0.08	5.90 ± 0.33	

Group 1: normal larvae developing on standard medium. Group 2: 'selected' larvae developing on standard medium containing 7% NaCl. Group 3: 'selected' larvae developing on standard medium containing 7% KCl.

Mean values and standard errors are given. The figures in parentheses are the numbers of different pooled samples on which determinations were carried out.

Table 2. *The composition of the media*

Medium	Osmotic pressure (mM./kg. NaCl)	Na (m-equiv./ kg.)	K (m-equiv./ kg.)	Cl (m-equiv./ kg.)
Standard medium	135	5	33	17.5
Standard medium + 7% NaCl	1400	1290	32	1230
Standard medium + 7% KCl	—	8	1050	1070

DISCUSSION

The haemolymph composition of normal larvae developing on standard medium shows features characteristic of pterygote insects. The Na:K ratio in the haemolymph is low. This feature can be associated with a phytophagous diet (Boné, 1944). The chloride concentration is low compared to the cation concentration. This anion deficit is regarded as divalent organic anion such as succinate and malate which occur in high concentration in the haemolymph of *Gastrophilus* larvae (Levenbook & Wang, 1948; Nossal, 1952). Ionized salts account for only a fairly small proportion of the total osmotic pressure of the haemolymph. This osmotic pressure deficit is probably mainly accounted for by amino acids, and Zwicky (1954) has in fact found a very high concentration of ninhydrin-positive substances in the haemolymph of *D. melanogaster* larvae.

The haemolymph of 'selected' larvae developing on standard medium containing 7% NaCl was markedly hypotonic to the medium. There is a rise of less than 40 mM./l. in the NaCl concentration. This increases the Na:K and chloride:organic anion ratios.

The haemolymph of 'selected' larvae developing on standard medium containing

7% KCl was also decidedly hypotonic to the medium. A small rise in the potassium concentration is more than compensated for by a fall in sodium concentration. This decreases the Na:K ratio to < 1 . The principal difference is that the chloride concentration has risen and the organic anion concentration has markedly decreased. This increases the chloride:organic anion ratio very considerably.

The external cuticle of *D. melanogaster* larvae is probably highly impermeable, but the larvae were constantly eating the concentrated media, and NaCl or KCl must have been rapidly entering the haemolymph across the gut epithelium. Well-developed mechanisms that can regulate the haemolymph composition must thus be present.

The regulation of body-fluid osmotic pressure in media with a high NaCl concentration is found in a few specialized types such as *Aedes detritus* larvae (Beadle, 1939) and *Artemia salina* (Croghan, 1958b). The ability with which these selected *D. melanogaster* larvae can regulate the haemolymph composition is thus remarkable. Selection for survival on a medium with a high NaCl concentration must have involved selection of a mechanism for the rapid excretion of NaCl. Gloor & Chen (1950) describe anal organs in *Drosophila* larvae. These consist of two silver-staining ventro-lateral plates with a thin cuticle and large underlying epidermal cells. They present evidence that these are concerned in salt uptake from hypotonic NaCl solutions. Waddington (1959) showed that these anal organs (papillae) were increased in area when the larvae were grown on media containing large amounts of NaCl, and that this increase was most marked in the selected larvae. It seems probable therefore that the anal organs are concerned in NaCl excretion in hypertonic media. The anal organs would function like the branchiae of *Artemia salina* (Croghan, 1958c). This mechanism would be distinct from that found in *Aedes detritus* larvae, where the area of the anal papillae is reduced compared to freshwater culicid larvae and where the mechanism is a specialization of the Malpighian tubule-rectal gland system involving the secretion of NaCl into the Malpighian tubule and the concentration of this by uptake of water in the rectum (Ramsay, 1950).

The ability of both normal larvae and 'selected' larvae to survive and develop on standard medium containing 7% KCl might seem even more remarkable. This is very different from the case of a type such as *Artemia salina* where a high KCl concentration is rapidly fatal (Croghan, 1958a). A very high potassium concentration is a feature of the Malpighian tubule fluid of insects. It has been demonstrated by Ramsay (1953a, b, 1955a, b) that the insect Malpighian tubule-rectal gland system operates in a cyclic manner. Potassium is actively transported into the tubule. This must be accompanied by an anion, which probably follows the potassium passively. In the proximal end of the tubule or in the rectum the potassium and anion are mainly reabsorbed. This cycle is associated with the circulation of water in the Malpighian tubule-rectal gland system (Ramsay, 1958). Given such a potassium cycle it seems clear that adaptation to a medium with a high KCl concentration could involve simply a decrease in the uptake side of the cycle. Potassium and accompanying anion would then be very rapidly excreted. Insects

would thus be expected to tolerate readily a medium with a high KCl concentration that would be fatal to other groups.

The fall in the sodium concentration in the haemolymph of *D. melanogaster* larvae on the medium containing 7% KCl suggests that normally the anion is actively reabsorbed, the cations following passively, as a decrease in the active reabsorption of anion in a medium with a high KCl concentration would then result in an increased sodium loss. The considerable fall in the organic anion concentration in the haemolymph of *D. melanogaster* larvae on the medium containing 7% KCl suggests that organic anion accompanies the active secretion of potassium into the Malpighian tubule, and that a failure to reabsorb this anion occurs in a medium with a high KCl concentration.

SUMMARY

1. The osmotic pressure, sodium, potassium and chloride concentrations have been determined in the haemolymph of normal *Drosophila melanogaster* larvae and of larvae selected to survive on standard medium containing 7% NaCl ('selected' larvae).
2. The haemolymph composition of normal larvae developing on standard medium shows features characteristic of pterygote insects.
3. The haemolymph of 'selected' larvae developing on standard medium containing 7% NaCl is markedly hypotonic to the medium. There is only a small rise in NaCl concentration.
4. Both normal larvae and 'selected' larvae can survive and develop on standard medium containing 7% KCl.
5. The haemolymph of 'selected' larvae developing on standard medium containing 7% KCl is markedly hypotonic to the medium. There is a decreased Na:K ratio and a very markedly increased Cl:organic anion ratio.
6. The nature of the mechanisms regulating the haemolymph composition is discussed.

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AN EXPERIMENTAL ANALYSIS OF THE FUNCTION OF THE PSEUDOBRANCH IN TELEOSTS

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I. INTRODUCTION

The pseudobranch is the remnant of the first gill arch in teleosts, situated antero-dorsally in the opercular cavity. In many fish it is buried beneath the skin and connective tissue, although in some species it hangs freely. The pseudobranch is absent in cyclostomes and elasmobranchs, and also in the Holocephali and Dipnoi. It is absent in a few teleosts such as the eel and some other eel-like fishes (Walls, 1942).

The pseudobranch is supplied with arterial blood from the first efferent gill artery, and within the pseudobranch this blood vessel is split up into a capillary system. Its efferent vessel (the ophthalmic artery) goes to the choroid gland of the eye (Grassé, 1958; Barnett, 1951) which is another capillary body. This chorio-capillaris is supplied secondarily by a branch of the carotid artery, the retinal artery, which supplies also the lentiform body of the eye. The venous blood from the choroid gland, along with blood from other parts of the eye, is drained into the venous channels of the head, and thus returns to the general circulation of the body.

The tissue of the pseudobranch consists almost exclusively of acidophil cells, which are of a general secretory type, thought to be the same or similar to those of the gills (the Keys-Willmer cells) (Copeland, 1951; Grassé, 1958). A recent electron-microscope study (Copeland & Dalton, 1959) clearly demonstrates that there are direct secretory ducts running between the cells and the channels of the capillary blood system. The cells are known to contain the enzyme carbonic anhydrase, and the pseudobranchs are the principal source of this enzyme in teleost fishes (Maetz, 1953; Vervoort, 1958).

The pseudobranch has had ascribed to it a number of functions, but none has been very clearly elucidated. The principal theories put forward are as follows:

- (1) That it is salt regulatory, since it contains Keys-Willmer cells.
- (2) That it is respiratory, functioning either as a supplementary gill, or in some way involving the enzyme carbonic anhydrase.
- (3) That it is an ocular regulator, either by controlling blood pressure in the eye, or by biochemically regulating the eye fluids via the choroid gland.
- (4) That it is an endocrine organ.

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II. MATERIALS AND METHODS

Experiments have been made on several species of marine and fresh-water fishes. These were the salmonids *Salmo trutta* (L.), as the fresh-water brown trout and as the marine sea trout, the rainbow trout *S. gairdnerii* (Richardson) in fresh water, the herring, *Clupea harengus* (L.), the saithe, *Gadus virens* (L.) and the plaice *Pleuronectes platessa* (L.) in sea water. The experiments were made in fresh-water conditions in the Freshwater Fisheries Laboratory, London, and in sea-water conditions in the Scottish Home Department's Marine Laboratory in Aberdeen. The fish were established for some time in these aquarium conditions before being subjected to any of the operational and experimental conditions. For the operation of pseudobranchectomy, the fish were anaesthetized with 'Metacaine' (Sandoz MS 222) in a concentration of 1:10,000. The pseudobranchs were removed surgically and the fish were replaced in the fresh or sea water from which they had come. Survival was almost complete, and very soon after such operations the fish were active and feeding. In some cases fish were 'mock-operated' by scraping the opercular lining, or by cutting filaments of the first gill arch; survival of both operated and mock-operated fish was similar, and any deaths were obviously the result of handling or of too deep anaesthesia.

III. EXPERIMENTS TO TEST THE SALT-REGULATING FUNCTION

The ability of operated and normal fish to osmoregulate was tested by subjecting marine and fresh-water fish to a series of sea-water dilutions and by following both their survival and the concentration changes in the blood. The survivals of control and operated fish did not differ significantly, and measurement of the freezing-point depressions of the blood at certain time intervals following the operation (by the method of Ramsay, 1949) showed that while the operated fish may initially demonstrate a wider variation in blood concentration, they did not show an impaired ability to osmoregulate after this initial period. The species used for this type of experiment in sea water were *Salmo trutta* and *Gadus virens*, and in fresh water *Salmo trutta* and *S. gairdnerii*. Some typical figures for such an experiment with the saithe are shown in Table 1. These results are also expressed graphically (Fig. 1) by plotting the mean values for internal blood concentrations against that of the external medium. It is clear that both normal and operated fish are able to control the blood concentration at the usual sea-water level, over a large range of external concentration.

Although the foregoing experiments made it clear that the pseudobranch was unlikely to have any direct osmoregulatory function, analyses of some common ions in blood and muscle were made on samples from operated fish (*Salmo trutta* and *S. gairdnerii* in fresh water) to see if the removal of the pseudobranchs had affected the regulation of specific ions. The operated fish used for these analyses had been maintained in aquaria for 9 months, in the same tanks with control, unoperated fish. The results of the analyses are shown in Table 2. No significant differences could be found in the levels of these ions in plasma or muscle extracts of operated and unoperated fish.

A further test of ion regulatory ability was made in experiments with the radioactive isotope of sodium, ^{24}Na . In these experiments fish of similar size were taken from aquarium stocks and injected with a dose of radiosodium, in the form of

Table 1. Osmoregulation in pseudobranchectomized and normal fish

(*Gadus virens* (saithe) from sea water and kept in sea-water dilutions. Freezing-point depressions ($\Delta^\circ\text{C}$) of blood.)

Medium ($\Delta^\circ\text{C}$)	0.48		0.96		1.44		1.92	
Time (hr.)	op.	n.	op.	n.	op.	n.	op.	n.
1	0.62	0.63	0.66	0.64	0.65	0.63	0.67	(0.69)
2	0.56	0.64	0.62	0.62	0.61	0.65	0.64	(0.69)
4	0.65	0.67	0.69	0.69	0.66	0.66	0.66	(0.69)
8	0.58	0.63	0.64	0.65	0.79	0.64	0.79	(0.69)
24	0.63	0.62	0.62	0.66	0.65	0.64	0.62	(0.69)
72	0.61	0.58	0.63	0.62	0.62	0.64	—	—
96	0.61	0.69	0.60	0.65	0.62	0.62	0.93	0.62

Mean $\Delta^\circ\text{C} = 0.69 \pm 0.02$ for a normal population of fish in sea-water aquaria. 'op.' are pseudobranchectomized fish. 'n.' are fish randomly selected from a natural population.

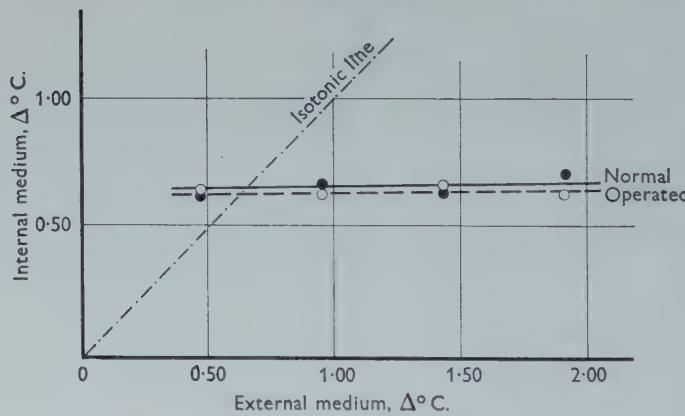


Fig. 1. *Gadus virens* (saithe) after 24 hr.

Table 2. Ionic regulation in pseudobranchectomized and normal fish

Sample	Ions (m-equiv./kg. water)			Ions (m-equiv./g. wet weight)		
	Na	K	Ca	K	Ca	Cl
<i>S. trutta</i> op. plasma	257	5.5*	2.6	—	—	—
<i>S. trutta</i> op. muscle	—	—	—	0.042	0.0024	0.0043
<i>S. trutta</i> n. plasma	264	6.6*	2.1	—	—	—
<i>S. trutta</i> n. muscle	—	—	—	0.042	0.0049	0.0096
<i>S. gairdnerii</i> op. plasma	266	1.9	3.6	—	—	—
<i>S. gairdnerii</i> op. muscle	—	—	—	0.058	0.0090	0.0058
<i>S. gairdnerii</i> n. plasma	235	1.1	2.0	—	—	—
<i>S. gairdnerii</i> n. muscle	—	—	—	0.050	0.0095	—

* Some laking of blood produces high values of plasma [K]. 'op.', 'n.' as in Table 1.

a normal saline, into the body cavity. They were then placed in tanks of sea water or fresh water and the rate at which the radiosodium was exchanged for non-active sodium measured by counting samples of the ambient water. A control tank of water with the same dose and the same volume was used. Thus two fish (one operated, one normal) of 150 g. weight each had a dose of 1 ml. saline and were placed in 50 l. water; the control tank of 50 l. water was similarly dosed with 1 ml. of the radioactive saline. The difference in counting rates of the fish tanks and the control tank was expressed as a ratio ('relative activity'), and thus calculations for decay rate were avoided. These experiments with *S. trutta* in fresh-water and sea-water conditions confirmed the survival experiments, since there was no evidence of a different rate of exchange of the radioactive ion in normal and operated fish (Fig. 2).

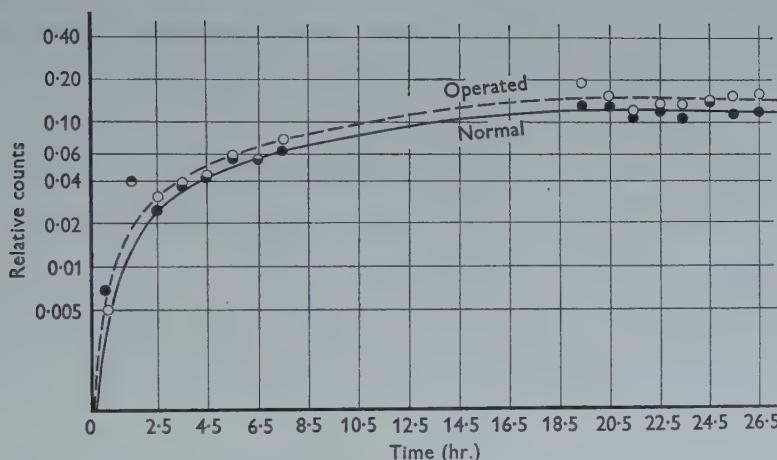


Fig. 2. *Salmo trutta* (brown trout) in static fresh water.

IV. EXPERIMENTS TO TEST THE RESPIRATORY FUNCTION*

Two types of 'respiratory stress' experiments were made, in which the survival times of operated and normal fish were observed. The method of treating the data obtained in such a survival experiment has been described by Alabaster, Herbert & Hemens (1957). The two experimental conditions were: first, a medium with high carbon dioxide and a moderate oxygen concentration; and secondly, a medium of low oxygen concentration but no carbon dioxide. The concentration of gases in each experiment was controlled at such a level as to provide a reasonable spread of results, too long a survival allowing some adaptation of the fish to the environment and too short a survival concealing any differences between operated and control fish, if they are present.

The ambient fresh water in these experiments was provided by a constant-flow

* These experiments were designed and made in collaboration with J. S. Alabaster, Freshwater Fisheries Laboratory, M.A.F.F., London.

apparatus, controlled by a flow-meter, and maintained at $20 \pm 0.5^\circ\text{C}$. throughout the experiment. The water was first forcibly aerated to drive off carbon dioxide and to aerate it fully. A part of this water was then bubbled with nitrogen so that it contained no oxygen. The oxygenated and deoxygenated waters so obtained were then mixed in fixed proportions using a dosing apparatus to provide for a flow of water of 0.7 l./min. The medium containing the high carbon dioxide concentration and moderate oxygen was similarly supplied by mixing three streams of water, one of which was saturated with carbon dioxide. The fish used in the experiments were small operated and control *S. trutta* and *S. gairdnerii* (4–5 cm.) and one experiment with larger specimens of *S. gairdnerii* (16–22 cm.). Ten operated and ten normal fish were used in each case, in a 40 l. tank for the larger fish and an 8 l. tank for the smaller ones.

The low-oxygen experiments had a concentration of 2.0 and 1.7 p.p.m. for the small fish, and 1.4 p.p.m. for the larger fish. The high carbon dioxide experiments had a concentration of carbon dioxide between 23 and 27 p.p.m. and an oxygen concentration of about 2.0 p.p.m. Oxygen levels were checked at 30 min. intervals by the Winkler method, and carbon dioxide at the same intervals by determining bicarbonate, pH and temperature and calculating the free carbon dioxide from the nomograms given by Dye (1952).

In the first set of experiments (high carbon dioxide), no consistent differences in survival of operated and control fish could be found (Table 3). The results of one such experiment, for rainbow trout, are plotted graphically in Fig. 3, and it is clear from these fuller results that although the median periods of survival of the two groups of fish in this experiment were different, there is no consistent separation of the survival of the two lots of fish.

Table 3. *The survival of pseudobranchectomized and normal trout at low concentrations of dissolved oxygen in the presence of about 30 p.p.m. carbon dioxide*

Species	Size (cm.)	Dissolved oxygen concentration (p.p.m.)	Median period of survival (min.)	
			Operated	Control
<i>S. gairdnerii</i>	4–5	2.1	47	41
	4–5	2.2	190	300
	4–5	2.2	40	100
	16–22	1.8	70	70
<i>S. trutta</i>	4–5	2.2	15	15

In the second set of experiments (in lethal oxygen concentrations) the results with both species of fish indicated that operated fish were able to survive considerably better than normal ones (Table 4). Figs. 4a and 4b express some of the full results graphically, and demonstrate the clear separation of the two groups of control and operated fish.

This difference in the survival of the operated fish is curious, since pseudobranchectomy might have been expected to reduce survival. It is suggested that

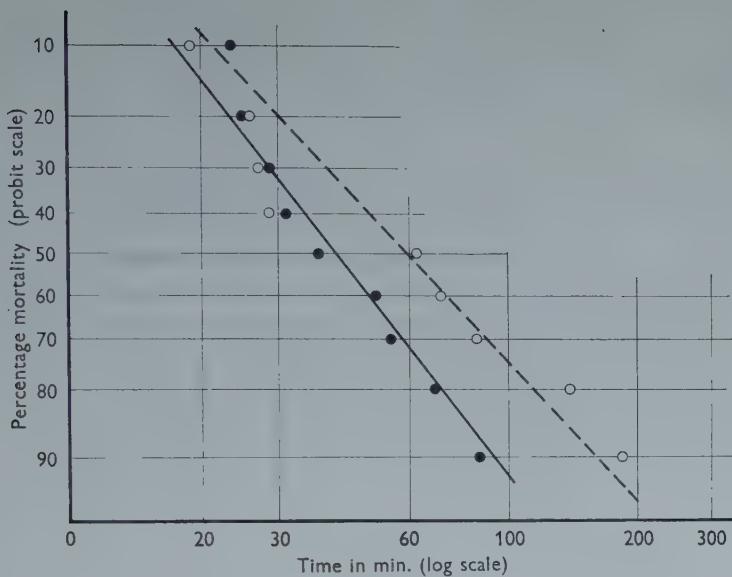


Fig. 3. Rate of mortality of rainbow trout in a concentration of dissolved oxygen at 2.0 p.p.m. and carbon dioxide at 20° C. ●, Normal; ○, pseudobranchectomized.

Table 4. The survival of pseudobranchectomized trout in lethal concentrations of dissolved oxygen

Species	Size (cm.)	Dissolved oxygen concentration (p.p.m.)	Median period of survival (min.)	
			Operated	Control
<i>S. gairdnerii</i>	4-5	1.7	170	145
	4-5	1.7	300	180
	16-22	1.4	64	45
<i>S. trutta</i>	4-5	2.0	59	28

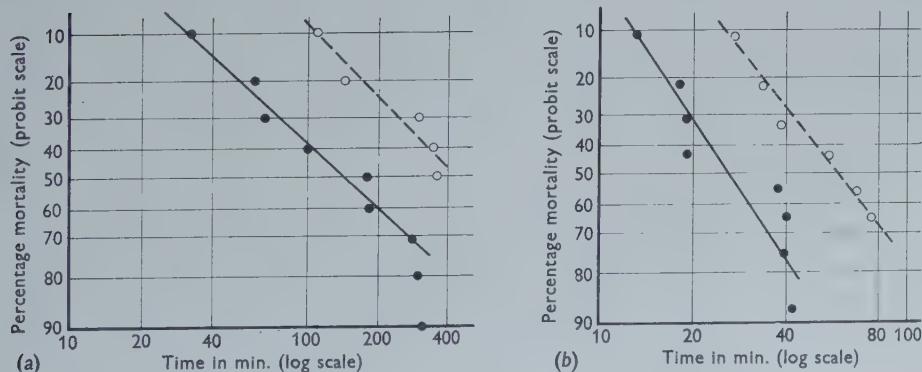


Fig. 4. (a) Rate of mortality of rainbow trout in a lethal concentration of dissolved oxygen at 20.5° C. (b) Rate of mortality of brown trout in a concentration of 2.0 p.p.m. dissolved oxygen at 20° C. ●, Normal; ○, pseudobranchectomized.

the higher survival value of pseudobranchectomy is brought about by a reduction in metabolic activity of the fish, following the operation. It had been observed that operated fish were quiet in aquarium conditions.

V. EXPERIMENTS TO TEST THE ENDOCRINE FUNCTION

During the course of these experiments, long-term observations (up to 15 months) were made on bilaterally and unilaterally pseudobranchectomized fish. Growth and maturation processes did not appear to be affected, although the general level of activity appeared lower in the operated fish than in control fish kept in the same aquaria. The most striking effect of pseudobranchectomy, which was evident within a short time of the operation, was the total and permanent darkening of the fish due to the maximal expansion of the chromatophores. This response was shown irrespective of light conditions or the colour of the background.

The time taken for the complete expansion of the chromatophores after pseudobranchectomy in brown trout was 12–15 min. Times of this order were also recorded for sea trout, saithe, flounders and plaice. In brown trout, expansion of the chromatophores had begun within 5 min. of the operation and the fish were visibly darker within this time. Certain areas of chromatophores responded more quickly than others: darkening began in the head region and the dorsal midline, and spread rapidly down the sides of the fish and then to the fins. The speed of this reaction is in marked contrast to that generally reported for such changes from white to black in intact fish (Parker, 1948).

It was further observed that unilateral pseudobranchectomy did not lead to darkening, nor did the incomplete removal of the glands. Cutting either the afferent or efferent blood vessels of the pseudobranchs on both sides of the fish also produced darkening, but not if the operation was on one side only.

It is well known that 'excitement pallor' in fishes can be induced as a shock reaction. In all our experiments care was taken to identify this shock pallor, which with careful handling and the use of anaesthetics was only a momentary effect, and to differentiate it from the response to the pseudobranch factor.

Mock-operated fish, or imperfectly pseudobranchectomized fish, served as controls for the darkening reaction; such fish remained light in colour in the usual laboratory light conditions. Any shock pallor following pseudobranchectomy was reversed very quickly.

In another series of experiments, the blood vessels to or from the pseudobranchs were ligatured with monofilament nylon. The disappearance of blood distal to the ligature could be clearly seen, thus demonstrating the effectiveness of the ligature. After ligaturing, the chromatophores of the fish expanded within 15 min. (in the brown trout) just as they did in pseudobranchectomized fish. If the ligature was released immediately after darkening had taken place, and the blood flow restored, the chromatophores returned to a contracted state within 30 min.; this change began to take place within 10 min. of releasing the ligature. If, as happened occasionally, the blood vessel was snapped in the process of tying or untying the

ligature, the dark phase persisted. If the ligature was left on for a longer period (about 1 hr.) the colour reversal was either very slow, or in some cases absent. This could indicate that the function of the pseudobranch had been impaired by a lengthy interruption of its blood supply.

Extracts of pseudobranch material were prepared by homogenizing freshly extracted or deep-frozen glands in saline solutions, and centrifuging to obtain a clear extract. Reinjections of such material were made into dark pseudobranch-ectomized fish. The potency of the extracts varied from one set of experiments to another. At the moment no explanation can be offered for this variation in potency. For instance, the reinjection of fresh brown trout pseudobranch material intramuscularly into dark operated brown trout in fresh water produced a local paling of the skin around the site of the injection. Pseudobranchectomized sea trout, which had been dark for some 6 months, returned to a light grey colour 3–4 hr. after intraperitoneal and intramuscular injection of a 1 ml. extract of 2 sea trout pseudobranchs. This paling was a temporary response and the fish became dark again within 24 hr. An extract of pseudobranch material made from the deep-frozen glands of horse mackerel and cod, when injected into dark plaice, produced local temporary paling and some reintroduction of pattern, within a period of 4–5 hr. Control operated fish were injected with a saline extract of gill-tip tissue and showed no such paling response.

The saline extract of horse mackerel and cod pseudobranchs was further tested for its effects on isolated chromatophores in fragments of the dark pectoral fins taken from pseudobranchectomized sea trout and plaice. This led to the complete contraction of both the melanophores and the erythrophores within 30 min. Control pieces of fin in a saline extract of spleen or in an extract of gill tips did not show this response within the same time period, or after a much longer period of observation (up to 6 hr.).

VI. DISCUSSION

It is appropriate here to consider some relevant anatomical and physiological facts concerning the pseudobranch. One of its most curious features is the blood supply, which is entirely arterial and thus fully oxygenated and presumably salt-regulated. It forms a capillary system within the pseudobranch, and after re-uniting to a single efferent ophthalmic vessel, forms a second capillary system in the choroid gland of the eye. This anatomical curiosity can be understood if the pseudobranch and the choroid are considered as a single unit. There is some embryological evidence for this (Goodrich, 1930) and further experimental support has been found in that pseudobranchectomy appears to be followed by atrophy of the choroid gland. Eyes from fish operated 9 months previously were found to lack the choroid gland; an alternative blood supply from the retinal artery exists to the eye. Similarly, extirpation of the eyes or keeping fish in the dark leads to a reduction in the size of the pseudobranch, and in the size of the acidophil cells (Pflugfelder, 1951). The absence of a pseudobranch in some teleost fishes (e.g. the eel and the catfish) is found to be associated with the absence of the choroid gland of the eye, and

conversely those fishes with a well-developed choroid (e.g. the horse mackerel) have large and prominent pseudobranchs (Walls, 1942).

The physiological evidence from the literature shows that the pseudobranch consists of secretory acidophil cells, producing either carbonic anhydrase or some substance involving carbonic anhydrase in its production, and secreting directly into the capillary vessels in the gland. Experimental evidence put forward in this paper establishes that an interruption in the blood supply of the gland, or the removal of the gland, brings about darkening in the fish. There is no evidence for any osmoregulatory or ion-regulating function of the gland, and no evidence of any direct respiratory effect. It may be concluded from these experiments that the pseudobranch is concerned in some way with the maintenance of the pale colour phase in normal teleost fishes. The nature of a possible chromatophore-concentrating substance in the gland calls for further experimentation.

On the basis of the experimental evidence put forward here it is possible to formulate an hypothesis which does much to explain our observations and is not inconsistent with any of the facts so far established.

The cells of the pseudobranch produce a substance, for convenience called '*P*'. This is released into the circulation and is carried via the efferent pseudobranchial artery to the choroid gland of the eye, and from there reaches the general circulation. '*P*' is a substance capable of stimulating the chromatophores to contract and the amount of '*P*' circulating in the body of the fish, in conjunction with other humoral and nervous mechanisms, determines its shade and pattern in relation to the background, by the responses of the chromatophores. Some of these chromatophores may have a lower threshold of response to '*P*' than others. The amount of '*P*' in the circulation is controlled by the state of the capillaries in the choroid gland; when fully dilated there is a maximal blood flow from the pseudobranch into the general circulation and the fish is pale; when the capillaries are fully contracted the circulation through the pseudobranch is restricted and the amount of '*P*' in the general circulation is thus low, and the fish is dark. Thus in this double system, the pseudobranch can be regarded as a self-replenishing reservoir of '*P*', and the choroid gland is the 'tap' which can be opened or closed to a varying degree. The control of the 'tap' might well lie in the amount of incident light falling on to the retina, with or without pituitary intervention. Fish in the dark, or blinded fish, are then dark because of the associated restriction of the amount of '*P*' released into the circulation.

This hypothesis and the observations on which it is based, recall the mechanism of colour control in the decapod Crustacea (Prosser, Brown, Bishop, Jahn & Wulff, 1950). Here the eye-stalk gland produces a hormone causing chromatophore contraction and removal of the gland results in maximum pigment migration. It seems likely that this gland in the Crustacea is in turn controlled by the light falling on the eye.

That the effects observed in fish are independent of nervous pathways is demonstrated by the experiments in which only the blood supply to the pseudobranch is interrupted. It might be argued that such experiments on the pseudo-

branch, by interrupting a major blood supply to the eye, produce results which can be, and have been, obtained by blinding the fish. However, if our hypothesis is accepted, blinding of the fish interrupts the blood supply from the pseudobranch, and thus prevents the circulation of 'P'. The experiments on isolated chromatophores demonstrate that the effect of the pseudobranch can be independent of the eye.

This suggestion that a new organ affects colour in fish is not incompatible with the existing interpretations of colour change. Indeed such an hypothesis for colour change in those teleosts possessing a pseudobranch, with or without pituitary influence, could clarify many otherwise confusing accounts of colour phenomena in the literature.

SUMMARY

1. The effects of removal of the pseudobranch have been studied in four species of marine fish and two species of fresh-water fish.
2. No evidence was found for any direct effect upon osmoregulation or respiratory exchange.
3. Removal of the pseudobranchs was followed by the darkening of the fish, due to chromatophore expansion, and after some weeks, by the degeneration of the choroid gland in the eye.
4. It is suggested that the pseudobranch produces, or activates, a hormone affecting the chromatophores and that the entry of this hormone into the general circulation is controlled by the choroid gland.

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ADDENDUM

The relationship between the activity of the acidophil cells of the gills and pseudo-branch of teleosts and some endocrine glands has been reviewed recently. Leiner (1938) and Leiner & Leiner (1940) held the view that the carbonic anhydrase produced by these cells in the various sites in fishes is of importance in the field of gas-metabolism. This has support from recent experimental work (Enami, 1959), at least for the cells of the gas-gland, although the function of the cells in the branchial region is not so clear. Enami reports that the cells in the gills of the catfish (*Parasilurus*) are stimulated to 'a kind of holocrine activity' by hypophysectomy, or by subcutaneous injection of eel urohypophysis, or by a number of chemical agents—sodium chloride, acetylcholine, adrenalin, noradrenalin, pilocarpine, or physostigmin. Transection of the spinal cord anterior to the urohypophysis, or implantation of excess pituitaries, leads to the disappearance of the cells. Thus there is some evidence that their activity is controlled by the urohypophysis, which is in its turn controlled by the pituitary. In *Fundulus*, on the other hand, Burden (1956) found no changes in the cells in the gills after hypophysectomy, although the mucous cells atrophied and the fish could no longer survive in fresh water.

The function of the acidophil cells in teleosts thus seems fundamental to their physiology, but the relationship to gas-metabolism, salt exchange and endocrine activity is confused and clearly requires further experimentation.

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SOME OBSERVATIONS ON TICK PARALYSIS IN MARMOTS

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Infestation with certain species of ticks gives rise in a number of animals to a paralysis which if unrelieved may end in death. The effect of such parasites is felt particularly in the ranching areas of South Africa and along the east coast of Australia, where sheep and dogs respectively are principally affected, and in cattle ranches west of the Rocky Mountains in Canada and the north-western United States. In this last area the condition is produced by the bite of the ixodid tick *Dermacentor andersoni* Stiles (Gregson, 1953).

The attachment of a single feeding female *D. andersoni* may also bring about a paralysis in man which again can have a fatal result if the causative agent is not recognized. Removal of the tick results in a dramatic reversal of the paralysis with no apparent after effects. The paralysis is of a flaccid nature, and exhibits a general ascending character of the Landry type in that the hind limbs are affected before the fore. As will be noted below, however, it seems that a rigorous sequence of development is not strictly followed. In addition to dogs, sheep, cattle and man *D. andersoni* affects guinea-pigs, hamsters and certain of the wild marmots and ground squirrels indigenous to British Columbia. Cats, rabbits, rats and mice show no symptoms in spite of the prolonged attachment of numbers of ticks.

Data on the nature of the defect resulting in the paralysis are meagre. Rose & Gregson (1956) showed that a peripheral neuromuscular block appeared to be present since electrical stimuli applied through motor nerves failed to give contractions while direct stimulation of the muscles was effective. This finding was confirmed in dogs by Murnaghan (1958a) and in marmots by Emmons & McLennan (1959). The latter authors showed also that the neuromuscular block was associated with a diminished release of acetylcholine. Murnaghan (1958b) originally reported that conduction in the motor fibres during the paralysis was normal, but recently has observed that in fact there is a failure of conduction in the motor nerves (Murnaghan, 1960).

Information regarding the causative agent produced by the feeding ticks is even less available. It has been established that the paralysis is not due to a viral infection (see, for example, Stanbury & Huyck, 1945) and that it is not an anaphylactic reaction seems likely since there is neither increased sensitivity nor immunity conferred by one exposure to the ticks towards subsequent infestations. The most probable explanation is that the tick secretes into or produces within its host a toxin which causes the paralysis, but that this material must be continually

administered since the symptoms may be fairly rapidly reversed on removal of the feeding ticks.

The investigation reported herein was undertaken as an extension of our earlier work concerning the failure of acetylcholine production in paralysed limbs brought about by motor nerve stimulation. We have now shown that the capacity of excised tissue from paralysed animals to synthesize acetylcholine is unaffected, and that there is a depressed conduction in peripheral motor and sensory fibres, in heart muscle and of transmission processes in the central nervous system. We suggest that the toxin produced by the ticks causes a generalized depression of excitability in all excitable structures.

MATERIALS AND METHODS

We have used marmots (*Marmota flaviventris avara* (Bangs)) as experimental animals. The ticks employed were collected in the vicinity of Kamloops, B.C., during the months of April and May, and were kept cool (*c.* 8° C.) until used.

Since it has been shown that the production of paralysis is dependent on the length of time which the ticks have been feeding (Gregson, 1958), they were fed for 5 or 6 days on sheep or rabbits before being transferred to the experimental animals. In this way the period of observation and tick protection was kept to a minimum, for two to six pre-fed female ticks produced paralysis with respiratory involvement in 24–36 hr. The ticks were confined to the marmots by capsules held to the abdomen by a band of adhesive tape, and protected further by placing each animal in a narrow cylinder of metal mesh.

Records of electrical activity from peripheral nerve were made with the aid of electrodes applied under light general anaesthesia. These were formed of dental cement with four silver wires projecting 0.5 mm., or small silver plates (0.2 × 2 mm.) on the surface of the plastic. The desired nerve was exposed, the perineurium removed, and a small bit of polythene tubing slit longitudinally passed around it. The electrode assembly was fitted within the tubing in close contact with the nerve and the muscle and skin was sewed over it. The assembly remained in place for at least 24 hr., as judged by the unchanged responses in normal animals. One pair of electrodes was connected to a square wave stimulator, the other to an amplifier and oscilloscope. Simultaneous electromyographic records were obtained with a concentric needle electrode inserted into the belly of the muscle. Usually the electrode assembly was applied to the main trunk of the sciatic nerve and the muscle electrode to the gastrocnemius. Records were obtained photographically. The electrocardiogram was recorded with a conventional instrument (Sanborn 'Visocardiette') with lead 2.

Acetylcholine synthesis by excised tissues was measured by the method described by McLennan & Elliott (1950). The main trunks of the sciatic nerves were incubated whole; transverse slices (1 mm. thick) of the spinal cord in the region of the lumbar enlargement and 1 mm. thick slices of cerebral cortex were also used. The tissues were incubated for 2 hr. at 37° C. in a bicarbonate-buffered medium equilibrated with 95% O₂-5% CO₂, and in the presence of eserine as an inhibitor of cholin-

esterase. The amount of acetylcholine produced by the tissue and liberated into the medium ('free') and that fixed in the tissue ('bound') were separately determined. Assays were performed by observing the depression of blood pressure of cats or rats.

RESULTS

Gross symptoms of the paralysis. The first sign of the developing paralysis in marmots was a loss of the animal's normal piercing cry, which was replaced by a hoarse grunt and appeared to indicate paralysis of the vocal cords. This was rapidly followed by an ataxia and weakness in the hind limbs, and the animal ceased eating. Thereafter the condition progressed to involve the fore limbs until the animal was unable to move and lay on its side. It was noteworthy, however, that even at this advanced stage of paralysis movement and tone in the tail persisted, while small movements of the neck muscles continued after respiratory distress was apparent. Voluntary eye movements were unaffected. There was no loss of consciousness, although it appeared that a considerable sensory deficit was present. Body temperature was maintained until the paralysis was well advanced, when it often fell abruptly (to c. 29° C.); throughout, however, the skin felt unusually cold and dry. There was retention of urine and faeces.

Following removal of the ticks from a marmot in which the muscles of respiration had been involved, there was often a continued deterioration in the condition of the animal with death the result. In those cases where complete recovery did take place it was slow and required considerable care of the animals (warmth, assisted respiration and feeding by stomach tube). In this respect marmots differ from man, dogs and other animals affected, in whom recovery is normally rapid and complete following removal of the ticks. Subsequent infestations of the same animals resulted in precise repetitions of the above sequence of events, and there was no change either in severity or in the time course of development of the condition.

Table 1. *Acetylcholine synthesis in vitro by tissues from normal and paralysed marmots*

	Acetylcholine ($\mu\text{g./g.}$) at end of 2 hr. incubation					
	Sciatic nerve		Spinal cord		Cerebral cortex	
	Free	Bound	Free	Bound	Free	Bound
Normal	1.1	3.9	0.8	3.7	2.0	3.1
	0.7	3.6	1.0	3.8	1.4	3.0
	1.3	3.3	1.6	3.2	1.6	2.6
	—	4.8	1.8	3.0	1.3	2.9
	—	—	—	—	—	—
Paralysed	1.5	6.0	1.9	4.7	3.4	—
	—	2.9	3.9	3.4	2.7	3.0
	1.4	4.6	3.3	—	0.8	2.3
	—	6.5	3.7	—	0.8	2.4

Acetylcholine synthesis in vitro. Table 1 sets out the results which were obtained when a comparison was made of the ability of peripheral nerve, spinal cord and cerebral cortex from normal and paralysed marmots to synthesize acetylcholine *in vitro*. It is evident that no diminished synthetic ability on the part of the tissues

from paralysed animals was found. The diminished output of acetylcholine following motor nerve stimulation in perfused limbs (Emmons & McLennan, 1959) is therefore not due to reduced synthesis of the substance.

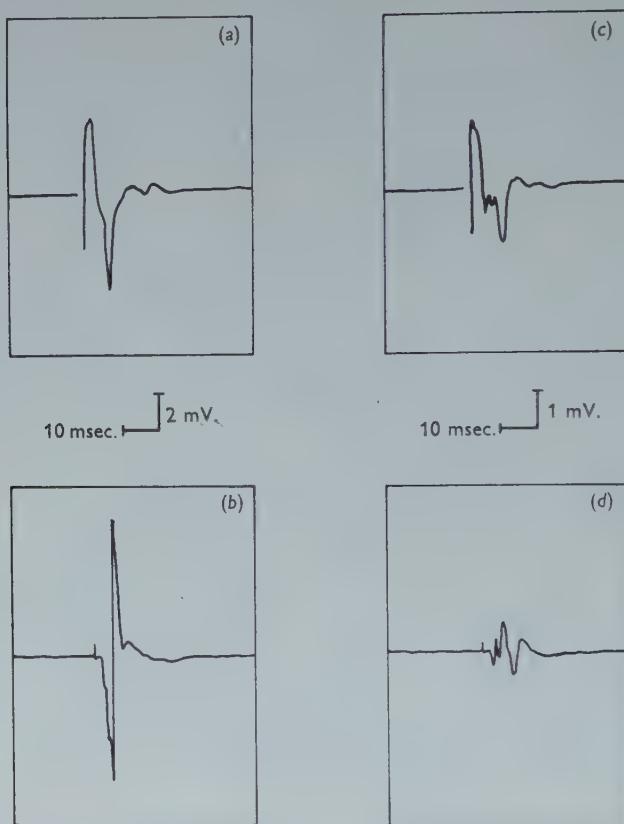


Fig. 1. Electrical responses of the sciatic nerve and gastrocnemius muscle elicited by maximal sciatic nerve stimulation, in a marmot before and after partial development of tick paralysis. Control (a) nerve and (b) muscle responses. Stimulus 7 V., 0.1 msec. Paralysed (c) nerve and (d) muscle responses. Stimulus 8 V., 0.1 msec.

Changes in conduction in peripheral nerve. Observations of the electrical responses recorded from the sciatic nerve when that nerve was stimulated, showed that marked changes had occurred in the paralysed state. The amplitude of all components of the compound action potential was reduced, which would indicate that conduction in both sensory and motor pathways was affected indiscriminately. Concomitantly there was a reduction in amplitude of the electromyographic response in the gastrocnemius muscle. These changes are illustrated in Fig. 1 by the responses of nerve and muscle before and after the development of partial paralysis in the same animal. In more severely affected cases the nerve and muscle responses were still further reduced in comparison with controls.

That conduction in both motor and sensory fibres was in fact depressed was demonstrated in acute experiments where the spinal roots were exposed. It has not been possible to compare the responses obtained in the same animal before and after paralysis had developed; nevertheless, the action potentials recorded in

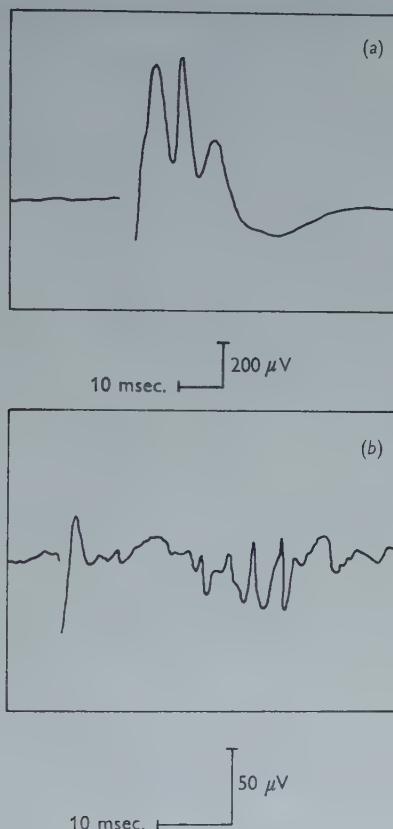


Fig. 2. Maximal reflex responses in marmots, obtained by stimulating a sensory root and recording from a motor root. (a) Normal, stimulus 8 V., 0.1 msec. (b) Paralysed, stimulus 10 V., 0.2 msec.

the sensory roots following sciatic nerve stimulation and those in the sciatic nerve as a result of motor root stimulation were invariably found to be much smaller than those obtained in control animals.

In view of this depressed conduction in both motor and sensory fibres it is somewhat difficult to assess the significance of the reduced reflex response recorded in motor roots upon stimulation of sensory roots, for it might be expected that the activity in the reflex arc would be small for this reason alone. However, almost no monosynaptic reflex response could be obtained at a time when there was still some conduction in both groups of root fibres (Fig. 2), and it would seem likely that the processes of transmission at the neurones of the spinal cord are affected during the paralysis as well as those of conduction in axons.

Effects on the heart. Changes in the electrocardiogram which we observed in paralysed animals fall into two groups. The first showed a sinus tachycardia in which the heart rate rose from its normal level of c. 190 per min. to c. 260 per min. The rhythm was regular and the intervals which have been measured were only

Table 2. *Electrocardiographic changes during tick paralysis in marmots*

	Average heart rate (beats/min.)	Intervals (sec)		
		P-R	QRS	Q-T
Normal	160	0.05	0.05	0.12
	170	0.07	0.03	0.11
	190	0.06	0.03	0.12
	220	0.05	0.04	0.12
	215	0.06	0.03	0.12
Average	190	0.06	0.04	0.12
Paralysed (tachycardia)	250	0.04	0.04	0.11
	250	0.06	0.05	0.09
	290	0.05	0.04	0.10
	260	0.06	0.04	0.12
Average	260	0.05	0.04	0.11
Paralysed (bradycardia and arrhythmia)	40	0.09	0.06	0.22
	90	0.10	0.06	0.27
	38	0.10	0.06	0.50
	75	0.09	0.05	0.26
	85	0.06	0.03	0.22
	85	0.06	0.04	0.26
	73	0.07	0.04	0.16
Average	70	0.08	0.05	0.27

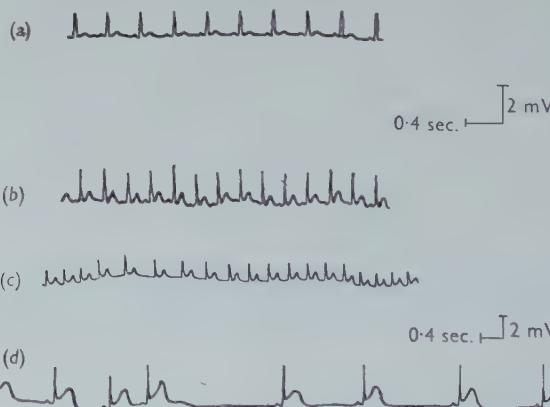


Fig. 3. Electrocardiographic changes during development of tick paralysis in marmots. (a) Normal; (b) tachycardia; (c) slight arrhythmia and bradycardia, intervals essentially unchanged; (d) severe arrhythmia and bradycardia, intervals prolonged and average heart rate very low. Note change in amplification in (a) and (b) compared with (c) and (d).

slightly below the normal (Table 2). The second group showed a pronounced bradycardia with some degree of sinus arrhythmia. The average rate fell to as low as 35–40 per min., with which was associated a much prolonged *Q-T* interval. The *P-R* interval was also somewhat extended in these cases (Table 2). Examples of these changes are shown in Fig. 3.

There were some indications that those animals showing arrhythmia were in a more advanced stage of the condition than those with tachycardia. It was noted above that the final stages of the paralysis were marked by a fall in body temperature and it was found that the deep temperature of all animals showing marked bradycardia was low, whereas those with tachycardia had a normal temperature.

DISCUSSION

Earlier work on the aetiology of the paralysis produced by the continued attachment of *D. andersoni* to experimental animals revealed that there was marked failure of neuromuscular transmission (Rose & Gregson, 1956; Murnaghan, 1958a) and that this was associated with a diminished release of the normal transmitter at the junctions (Emmons & McLennan, 1959). Murnaghan (1958a) deduced that the block was localized at the pre-junctional endings. The present experiments have demonstrated that there is an unimpaired ability of the tissues in paralysed animals to synthesize acetylcholine, from which it may be concluded that the absence of acetylcholine in the perfusate following stimulation may be due to a depressed release of the transmitter or to a failure of motor nerve conduction.

Other experiments described here show that there is in fact a diminished conduction in the spinal motor roots and hence in the peripheral motor nerve fibres. This finding adequately accounts for the flaccid motor paralysis which is the most pronounced symptom of the condition.

We have also shown that in marmots there is a loss of conduction in sensory fibres. This is borne out by the gross observation that paralysed animals are much less responsive to a painful stimulus than normals. Reports of the condition in humans on this point are far from consistent. Where changes in sensation have been specifically looked for or reported, the majority of cases are said to show none; however, numbness, paraesthesia and even complete sensory anaesthesia have been reported in a few instances (see, for example, Mail & Gregson, 1938; Stanbury & Huyck, 1945). In most human cases the condition does not progress to the extent usual in our experimental animals, and it may be that in these cases subjective sensory loss occurs later than does the motor defect and is not marked at the time of removal of the ticks. We have no experimental evidence to decide whether this is a true explanation or not.

The changes in the electrocardiogram observed during paralysis are not easy to interpret. Sinus tachycardia may occur in many toxic conditions. There is no indication of a defective conduction of impulses in the heart muscle at this stage. The sinus arrhythmia with bradycardia which develops later, with which is associated lengthened *P-R* and *Q-T* intervals, would indicate a depressed conduction and a slower rate of auricular and ventricular depolarization and repolarization. These results, then, are not inconsistent with the depressed conduction found in peripheral nerve. It should be noted that the defective circulation in late stages of the paralysis cannot be the cause of the condition, for all animals show complete paralysis of all four limbs before arrhythmia develops. The effects on the

heart therefore represent another manifestation of the widespread action of the toxin.

It is not possible on the basis of our experiments to decide absolutely whether the spinal neurones involved in the monosynaptic reflex arc are also depressed, or whether the areflexia is to be attributed entirely to the reduced conduction in the sensory and motor roots. The former would seem to be the case in instances like that illustrated in Fig. 2. It is reasonable by analogy with our other findings that a slower rate of depolarization of the neuronal soma and a longer absolute refractory period might be present, and that these contribute to the absence of activity in the reflex arc. Future experiments will be directed towards establishing this point.

In conclusion, the results which we have described show that the toxin produced by feeding ticks affects more structures than those of the motor pathways, and that all such structures show a lowered excitability. The effects of the heart indicate that the rates of depolarization and repolarization are slowed. It is possible that this may be the underlying cause of all observed changes and that all excitable tissues are affected to some extent.

SUMMARY

1. Observations have been made on marmots paralysed by the attachment of the ixodid tick *Dermacentor andersoni* Stiles.
2. Acetylcholine synthesis by excised tissues from paralysed animals is unaffected.
3. Conduction in both motor and sensory nerve fibres is markedly reduced. It is likely also that the excitability of neurones of the spinal cord is diminished.
4. There are changes in the electrocardiogram suggestive of a slowed rate of auricular and ventricular depolarization and repolarization.

We are deeply indebted to Mr J. D. Gregson, Entomology Laboratory, Kamloops, B.C., for his continued great interest in this problem and for supplying us with marmots and with ticks. We wish also to thank Mr A. J. Honour for his assistance with many of the experiments.

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THE SHADOW REACTION OF *DIADEMA ANTILLARUM* PHILIPPI

I. THE SPINE RESPONSE AND ITS RELATION TO THE STIMULUS

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INTRODUCTION

Though many animals respond to shadows some do so without clearly defined photoreceptors, and in such cases detailed studies of the mechanism involved are rare. Instances of this kind have been investigated by von Buddenbrock (1930) and Föh (1932) who studied, in particular, *Balanus* and *Helix*, respectively.

The echinoid *Diadema*, previously examined by von Uexküll (1900) and Millott (1954), is another example, responding to changes in illumination by movements of its spines, podia and pedicellariae. Of these, spine movements are the clearest and most consistent, and we have studied them exclusively. They are reflexes, the pathways of which pass through the radial nerves.

Most of the body surface is light-sensitive, though morphologically defined photoreceptors have not yet been found and sensitivity is co-extensive with the nervous system, much of which is epidermal. This suggests that relatively unspecialized elements may be directly excited by light, and this has been strengthened by a direct demonstration of photosensitivity in the radial nerves (Yoshida & Millott, 1959).

In reactions to shadows, both the illumination and its change must be considered, since both are environmental agents. We therefore set out to discover what effects varying the intensity and duration of both lighting and shade exert upon the character of the spine response.

METHODS

Urchins from Madeira were used and they were kept in aquaria.

The urchin was cut into five pieces, each with a radial nerve cord at the centre. After removing viscera, each piece was carefully washed with fresh sea water and the spines were removed by cutting as closely as possible to the test, leaving one in a position slightly aboral to the ambitus. The piece was mounted horizontally in aerated sea water contained in the experimental tank already described (Millott & Yoshida, 1957), and left to recover and adapt for at least 45 min. before use. The inside of the tank was painted matt black, apart from two narrow slits on either side which allowed a light beam to throw a shadow of the spine tip on to a ground-glass screen. Spine movement was recorded photographically in the way already described.

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Light for stimulation was obtained from a 6 V. 30 W. tungsten filament lamp run on a.c. except where shadows shorter than 0·3 sec. were used, when it was operated on d.c. to avoid flicker effects. The voltage was kept constant by means of a variable resistor and voltmeter. Intensity was controlled by interposing neutral filters.

To minimize the spread of stray light and the complicating effects of spatial summation, the areas illuminated were kept as small as possible by projecting a beam through the objective lens of a microscope, as already described (Yoshida & Millott, 1959). The beam was focused on to the centre of the radial nerve.

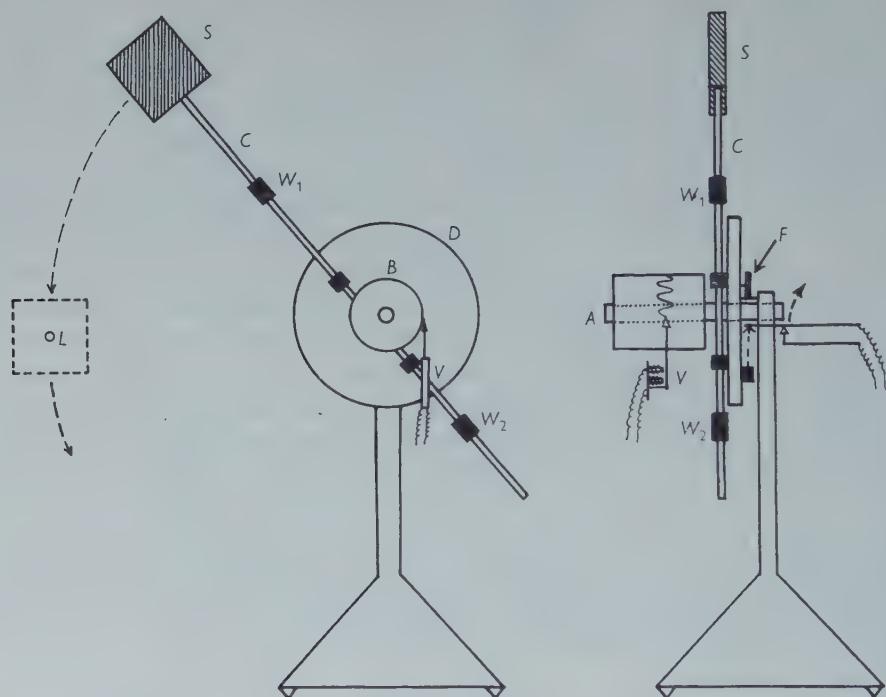


Fig. 1. The apparatus for producing brief shadows. For description see text. *A*, axle; *B*, kymograph drum; *C*, calibrated shaft; *D*, wheel; *F*, stop; *L*, cross-section of light beam; *S*, opaque shutter; *V*, vibrator; *W₁*, *W₂*, weights.

However, when brief shadows were used a slight modification was required to produce them by a shutter rotating in a vertical plane (see below), so that the stimulating light had to be mounted horizontally and its beam deflected into the optical system, by means of a 45°-prism.

Shadows longer than 0·3 sec. were produced by manually operated shutters, the exact duration of shading being recorded photographically and measured by an adjacent time trace.

Various means of producing shorter shadows were tried. Of these the most consistently satisfactory is shown in Fig. 1. The axle *A*, rotating on ball bearings,

carries a kymograph drum *B* and wheel *D* to which is bolted the calibrated shaft *C*. This carries the opaque shutters and sliding weights *W*₁ and *W*₂. The beam from *L* was interrupted by the shutter each time the shaft and its attachments moved under its own weight from the constant position ensured by a stop in the wheel, indicated in the figure by *F*. Different durations of shadow were obtained by altering the position of the shaft, the weights and the length of the shutters. The speed with which the shutter passed over the light beam was determined for each setting of the apparatus by recording the speed of rotation, using the trace from a 100-cycle vibrator (*V*) writing on the drum surface.

Each record showed spine movement accompanied by a time trace (at 1 sec. intervals) and an automatic signal showing when the light beams were cut off. In recording the initial part of the reaction, the paper was run at 0.5 in./sec., which gave reasonable accuracy in calculating the reaction time and duration of shading. For the sake of economy, this speed was slowed down to 0.1 in./sec., 6–8 sec. after the beginning of the shadow. Examples of the recording are shown in Fig. 2.

Experiments were performed in a dark room the temperature of which was fairly steady, so that in any series of experiments the temperature of the sea water did not vary by more than 1° C.

The spine response

Responses appear consistently in pieces of test prepared as described above, and if adequate time for recovery is given the spines are motionless until stimulated, so that both beginning and end of a reaction are easily determined. Though spontaneous movements may occur, they are relatively rare.

Movement follows stimulation after a well-defined interval and lasts for a few seconds, or even a minute, declining gradually in vigour. Whether stimulation be general or localized, it affects most, if not all, of the spines so that extensive co-ordination is involved. The interpretation of such movement is difficult, so to simplify matters we directed attention to one spine and removed the remainder.

With the records obtained, reaction time, amplitude, frequency and duration of the contractions can readily be measured and compared. The reaction time is defined as the interval from the beginning of shading until the first signs of a response; the frequency as the number of beats recorded during a standard period after the beginning of stimulation; and the duration as the period from the beginning of shading until all contractions have finished. The amplitude is the total swing of the spine as it appeared on the ground-glass screen, expressed in arbitrary units. In comparing reactions we have used both the amplitude of the first contraction (initial amplitude) and that of the largest contraction occurring after 10 sec. from the time of shading (later amplitude).

These criteria can be used to compare reactions because they are reasonably constant in response to a constant stimulus, so that records taken within 1–2 hr. are often so similar as to be almost superimposable (Fig. 2).

However, as the preparation ages the vigour of response may decline gradually (Fig. 3), particularly in the later part of the reaction, but the reaction time stays

fairly constant, even when the response has become so weak that only few jerks ensue after a stimulus which earlier called forth a vigorous reaction. This means that when determining the effect of environmental factors on the vigour of the response, control experiments had to be carried out frequently.



Fig. 2. Reactions to a shadow of infinite duration and a constant intensity of 100% (see p. 369), shown by one preparation, to illustrate the constancy of the response. Records of three separate reactions, recorded at approximately hourly intervals, have been superimposed in the left half, which represents the more critical part of the reactions whose characteristics are compared. The interruption of field illumination is shown by the change in level of the line above the time trace. Time in seconds.

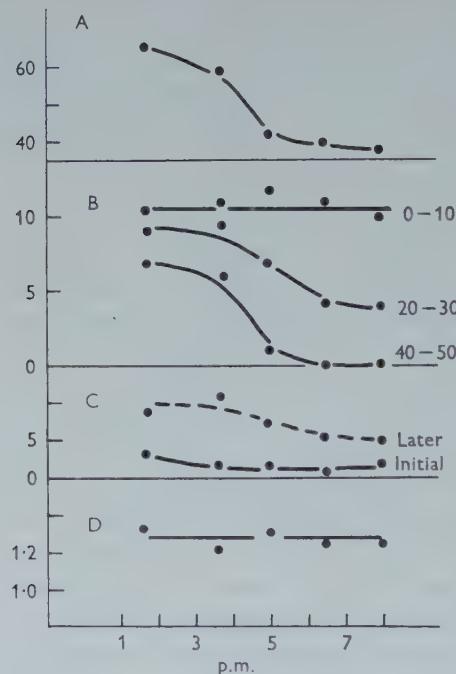


Fig. 3. The decline in vigour of the response. Abscissae, time of day. A, duration. Ordinates, time in seconds. B, frequency of contraction. Ordinates, number of beats in 10 sec. interval indicated alongside curve. C, amplitude of contraction. Ordinates, arbitrary units. D, reaction time in seconds.

Although spine responses follow shading of both the radial nerve and the skin, we have preferred to study the effect of the former because in the case of the outside surface the reaction times which follow shading at various places along a plane parallel to the ambitus vary considerably, as shown in Fig. 4, and a shift of only 2.0 mm. (from the ambulacral margin to the adjoining interambulacrum), implies very different reaction times. The variation is much less in the radial nerve, the

mean and its standard deviation in different positions ranges from 1.22 ± 0.04 to 1.32 ± 0.06 sec.

It may be noted in passing that the variation in reaction time at various points on the outside surface corresponds with the relative sensitivity of these areas, previously determined by a different method (Millott, 1954); the most sensitive areas, having the shortest reaction time, are found at the ambulacral margin and a gradient in sensitivity and reaction time exists as follows:

ambulacral margin → ambulacral centre → inter-ambulacrum.

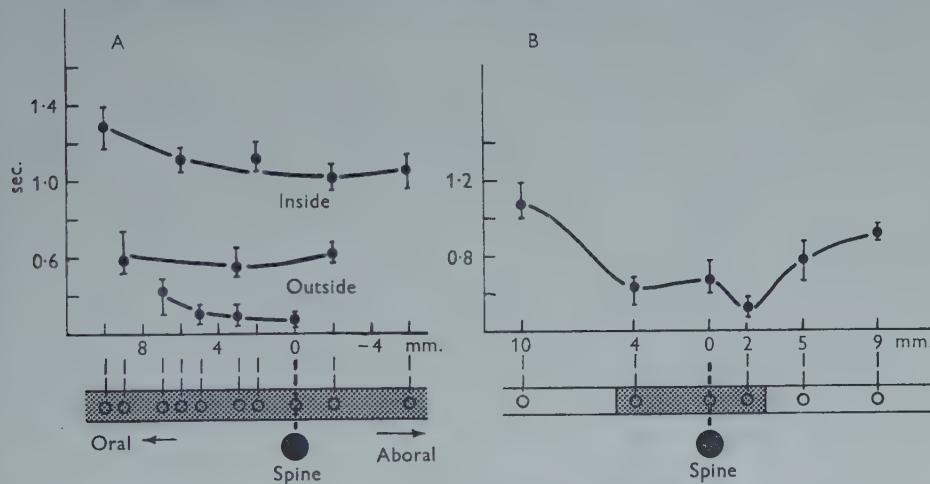


Fig. 4. The reaction times of the responses elicited by shading various positions internally and externally. Each curve corresponds to one preparation. Ordinates, reaction time in seconds, the vertical bar showing the range of variation at each point. Abscissae, the numbers show the approximate distance in mm. of the area stimulated from a position 0 which lies alongside the spine, as shown in the accompanying diagram where the ambulacral areas appear stippled. A, meridional gradient. The curve marked 'inside' shows the effect of stimulating internally by shading positions lying along the radial nerve, that marked 'outside' showing the effect of shading positions on the outside surface, which lie along the margin of the ambulacrum. B, the effect of shading the outside surface in various positions which lie across the ambulacrum, in a plane parallel to the ambitus.

The effect of illumination

To show this we determined the effect of varying the duration and intensity of illumination, while the degree of shading was kept constant by cutting off the light completely and leaving the preparation in darkness, at least until the reaction had subsided.

It was necessary to guard against possible effects of sensory adaptation by interspersing experiments in which the field illumination was dim between those in which more light was used.

(a) Duration

The effect of this was determined by illuminating preparations at a constant intensity for periods between 1 and 300 sec., after which the field illumination was

cut off. The preparation then remained in darkness for not less than 5 min., after which another experiment was begun. Such intervals had been found adequate to allow full recovery of responsiveness.

The results from one preparation are shown in Fig. 5, where it will be seen that the reaction time decreases steadily as the duration of lighting is increased. In contrast, the frequency of the contractions and the duration of the reaction increase steadily as the illumination is prolonged. The changes are complete after $1-1\frac{1}{2}$ min. illumination, beyond which no further significant increase in responsiveness appears. The amplitude of both initial and later contractions shows a similar tendency.

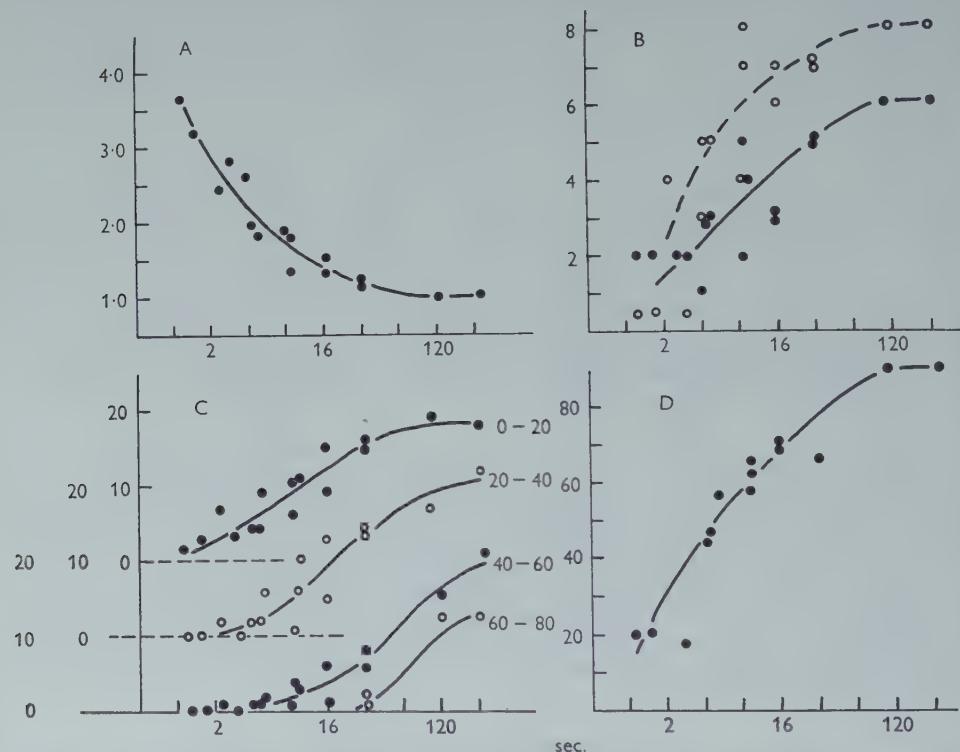


Fig. 5. The effect of the duration of lighting on one preparation. Abscissae, duration of lighting in seconds. Ordinates: A, reaction time in seconds. B, amplitude in arbitrary units. Filled circles, initial contraction; open circles, later contraction (see p. 365). C, number of beats recorded in the 20 sec. period indicated alongside each curve. D, duration of reaction in seconds.

(b) Intensity

A complementary series of experiments was performed in which the light was cut off after a 5 min. exposure to different intensities with a range of 10^3 . The results from one preparation are shown in Fig. 6.

The effects on reaction time, amplitude, frequency and duration, produced by increasing the intensity, are essentially similar to those produced by prolonging the lighting.

These experiments also revealed that the reactions of the primary spines vary with intensity in the same way as those of the spines of lower orders, so that possible differences in the effectors are not important here.

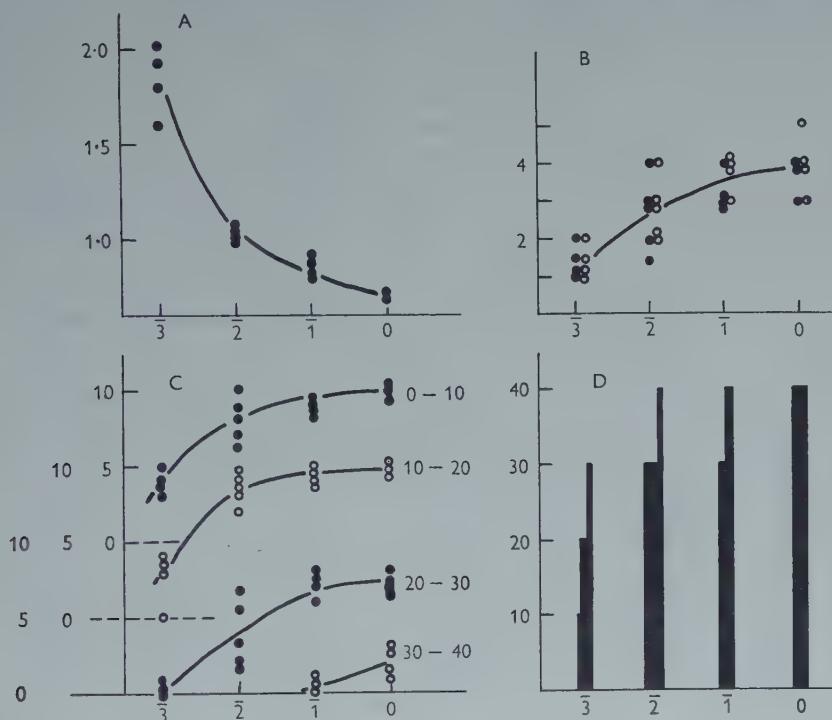


Fig. 6. The effect of intensity of lighting on one preparation. Abscissae, logarithmic scale in arbitrary units. Ordinates: A, reaction time in seconds. B, amplitude in arbitrary units. Filled circles, initial contraction; open circles, later contraction. C, number of beats observed in the 10 sec. period indicated alongside each curve. D, duration of the reaction in seconds. The height of each shaded area represents the 10 sec. period during which the reaction subsided. The number of reactions whose duration is recorded, is represented by the relative width of the areas shaded.

The effect of shading

The effect of a shadow may now be examined in the same way, its intensity being considered as the decrease in intensity of field illumination and its duration as the interval between the instant it is reduced and the time that the preparation is reilluminated. The field illumination was kept constant, preparations being illuminated at the same intensity for 5 min.

(a) Duration

Here the field illumination was cut off completely for periods varying between 26 msec. and about 1 min., a period of about 1 min. being the time taken for completion of the reaction.

Where the duration of shading is shorter than that of the reaction the increase in intensity due to the light re-admitted may superimpose an 'on' effect. The animal being relatively insensitive to such changes (Millott & Yoshida, 1959), this danger was avoided by working with an intensity of field illumination well below the threshold for such responses.

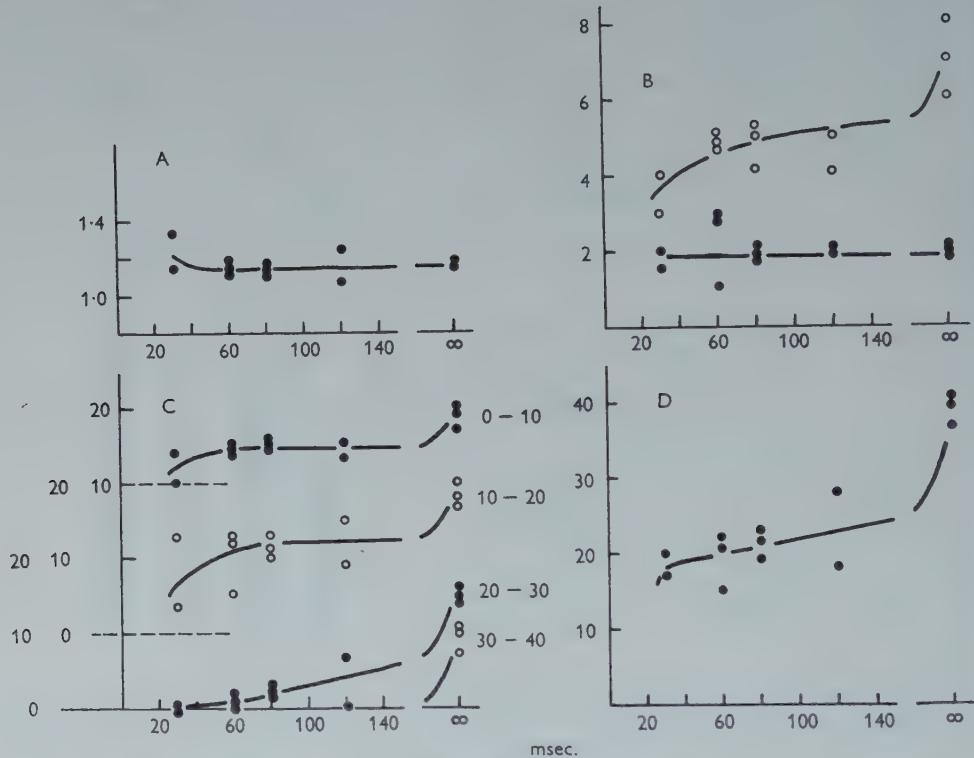


Fig. 7. The effect of shadows up to 120 msec. duration compared with that of a control shadow of infinite duration. From a single preparation. Abscissae, duration of shadow in milliseconds. The results of the control are shown alongside. Ordinates: A, reaction time in seconds. B, amplitude in arbitrary units. Filled circles initial contraction; open circles, later contraction. C, number of beats recorded during the 10 sec. period indicated alongside each curve. D, duration of the reaction in seconds.

Typical results, reproduced in Figs. 7 and 8, show that there is little or no effect on the initial part of the reaction, but more on the later parts.

Thus the amplitude of the first contraction is unaffected and the reaction time is affected only near the threshold, i.e. below 40 msec. and then but slightly and erratically.

The effect on the later part of the response involves the amplitude, frequency and duration, all of which increase with the duration of shading. The increase is not uniform, being greater as the shadow increases up to about 60 msec. (Fig. 7). Prolonging the shading further brings about relatively little change, except in the

case of the frequency of the contractions occurring later in the reaction (after about 20 sec.), which increase more significantly. In all features, with the notable exception of the reaction time and initial amplitude, the reaction is smaller after light has been re-admitted, regardless of whether this occurs before or after the reaction has begun.

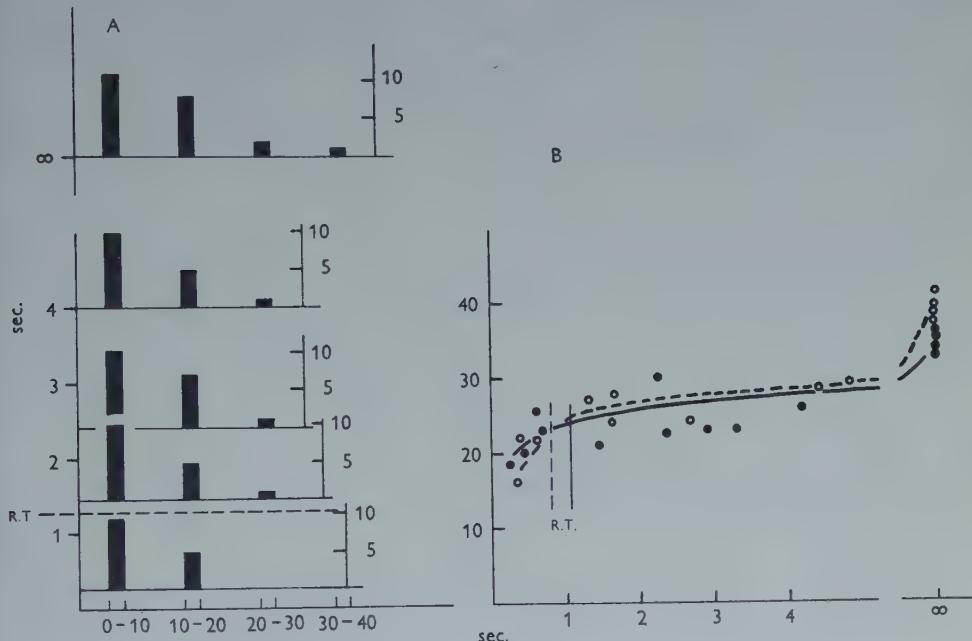


Fig. 8. The effect of shadows 0.3 sec. or longer in duration compared with that of a control shadow of infinite duration. A, effect on the number of beats in a single preparation. Abscissae, successive 10 sec. periods. Ordinates right, the number of beats recorded in each 10 sec. period. Ordinates left, the duration of the shadow. The vertical height of each shaded area represents the number of beats recorded in each 10 sec. interval. Each shaded area is displaced along the ordinate axis so as to show the duration of shadow which produced this effect. The average reaction time (R.T.) is shown by the broken line. B, effect on the duration of the reaction; abscissae, duration of shadow in seconds. Ordinates, duration of reaction in seconds. Open circles and broken line, filled circles and continuous line, represent two different preparations. The reaction time is shown by the corresponding vertical lines.

(b) Intensity.

To show the effect of intensity, neutral filters of different densities were interposed in the light beam after 5 min. and allowed to remain until the reaction subsided. The results are shown in Fig. 9, the intensity of shading being expressed as a percentage decrease in field illumination.

They differ from the preceding in that the reaction time decreased steadily as the depth of shading was increased. For other criteria, the shortage of material compelled us to confine our measurements to two preparations, which showed that the duration of the reaction and the frequency of the beats increased with the

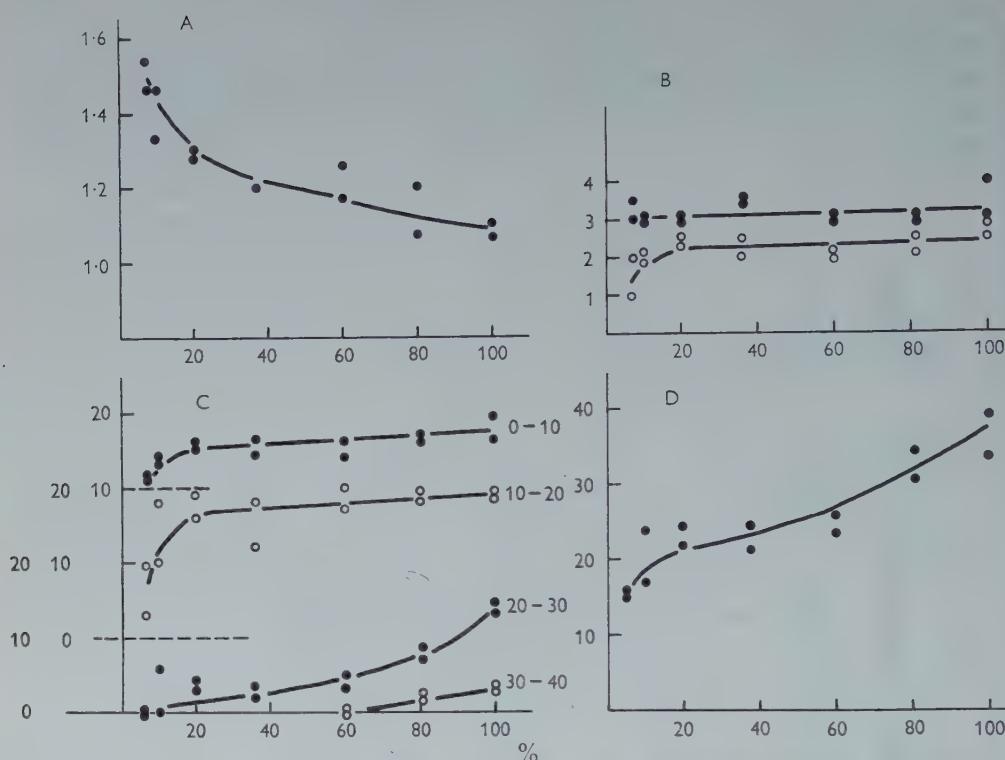


Fig. 9. The effect of the shading intensity (p. 369) on one preparation. Abscissae, percentage decrease in intensity. Ordinates: A, reaction time in seconds. B, amplitude in arbitrary units. Filled circles, initial contraction; open circles, later contraction. C, number of beats recorded in the 10 sec. period shown alongside each curve. D, duration of the reaction in seconds.

intensity of shading; in addition, the amplitude of the later contractions was increased and in some cases that of the initial contractions also.

DISCUSSION

Very little is known of the neuromuscular organization of echinoids, so that the conclusions which can be drawn from an analysis of a gross response such as spine movement must necessarily be limited. As compared with an analysis by electrophysiological means the approach not only lacks precision but is hampered by the fact that one sees only the terminal event in a chain involving various responsive structures, receptive, conducting, interacting and contracting. But despite the resultant complexity, some significant relationships emerge between stimulus and response. These are summarized in Table 1.

The reaction time, frequency, amplitude and duration of the response are clearly related to both the intensity and duration of the preceding field illumination.

Their relationship with the shadow is different, for its intensity affects the reaction time (and in some cases the amplitude of the initial contractions) but its duration does not.

Table 1. Effects of light and shade on various criteria

(+ = clear effect; - = no effect; ± = doubtful effect.)

	Criterion	Field illumination		Shadow	
		Duration	Intensity	Duration	Depth
Initial part of reaction	Reaction time	+	+	-	+
	Amplitude	+	+	-	±
	Number of beats	+	+	±	±
Later part of reaction	Amplitude	+	+	+	±
	Number of beats	+	+	+	+
	Duration	+	+	+	+

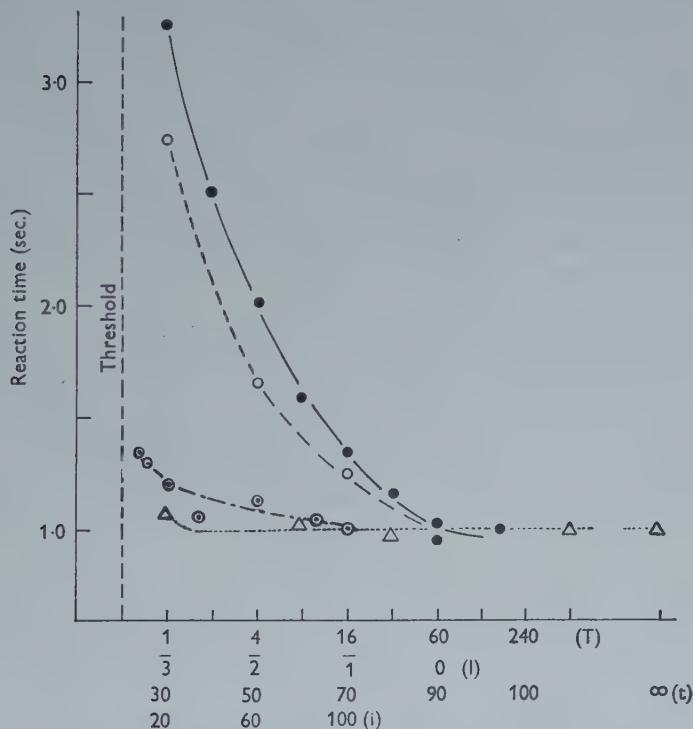


Fig. 10. Comparison of the effects of lighting and shading on the reaction time. Curve T duration of lighting, ●—●. Curve I intensity of lighting, ○—○. Curve i intensity of shading, ○·—○·. Curve t duration of shading, △··△. Abscissae: (T), in seconds; (I), in arbitrary logarithmic units; (i), percentage decrease in field intensity; (t), in milliseconds.

The relation between stimulus and reaction time is especially significant for here the different effects of illumination and decrease in illumination appear (Fig. 10). The duration of the field illumination affects the reaction time, that of the shadow does not, and so the expected relationship between intensity and duration of the stimulus does not appear in the case of the shadow.

However, the duration of the shadow affects the later part of the reaction, which means that it differs from the initial part in its variability and susceptibility. This

might imply that an additional mechanism comes into play in the later reaction (see below).

Although these findings resemble those of von Buddenbrock (1930) and Föh (1932)—both of whom used a similar index, namely the terminal response of a reflex arc—there are significant differences. Also it must be remembered that the methods differ, for they used intact animals whereas we used spines isolated from other similar effectors, and the areas illuminated and shaded were far greater than those used here.

In *Helix* Föh found the intensity of field illumination and shading to affect the reaction time, vigour and duration of the response in essentially the same way as in *Diadema*, but the effect of the duration of shading was different in that it affected not only the vigour and duration of the reaction, but also the reaction time, provided that the duration of shading was within a critical range, viz. 240 msec.

The lack of an effect of duration of shading on the reaction time, except when the duration is near threshold, leads us to interpret our results in a different way.

The parallel effects of duration and intensity of field illumination are such as to suggest that it is here that light acts in conditioning a system which is 'set' thereby, and remains so until overt effects are released by the decrease in intensity, the duration of which has no effect on the early part of the response.

The fact that under the conditions in the experiments described above steady light produced no overt effects suggests that it may be exerting an inhibitory effect, release from which is provided by the shadow and that the reaction which ensues is roughly in proportion to the effect of the preceding illumination, as well as to the intensity of light remaining during shading, which continues to exert an inhibiting effect. Then it would be evident why the intensity of shading is important; for, being a measure of the decrease in intensity, it is also a measure of the intensity of the light remaining. Further, when light is re-admitted inhibition might again affect the reaction, diminishing or cutting it short according to the moment at which light is re-admitted. The significant timing would therefore be that of the re-admitted light and its ineffectiveness on the initial part of the reaction could thus be an expression of the latency of the inhibition.

This possible explanation at once recalls that usually adduced to explain the 'off' effect in eyes (Granit, 1947), which involves nervous interaction. In this connexion it is pertinent to recall the work of von Buddenbrock, who showed the effect of the intensity and duration of shading on the duration of the withdrawal reaction of *Balanus*, which he further states does not depend on the intensity of field illumination, though he shows that the duration of the threshold stimulus depends on it. He also showed the existence of spatial and temporal summation in the shadow response. Most significantly, this led him to argue cogently against much current opinion, notably that of Puettner and Hecht, and to emphasize the importance of the central nervous system. Föh, on the other hand, on a basis of a quantitative study of the relationship between duration and intensity of shading, made a comparison of the effect of a shadow with that of illumination in the formal

scheme advanced by Hecht. As already mentioned, our different results have led us to reject Föh's approach.

To strengthen our suggestion concerning the participation of inhibition, it is desirable to demonstrate by direct experiment, the inhibitory action of light on the shadow response. This will be shown in a succeeding communication.

SUMMARY

1. Isolated pieces of test of *Diadema* bearing a single spine show responses to a constant shadow cast on the radial nerve which are consistent in reaction time, duration, amplitude and frequency of the contractions.
2. Variations in the intensity and duration of the lighting which precedes the shadow exert similar effects on the whole reaction, affecting all the above features.
3. Variations in the duration and intensity (% decrease in intensity of illumination) of the shadow differ in their effects. The intensity affects all the features of the whole reaction, but the duration affects only the later part.
4. The differing effects of light and shade suggest that the shadow response may be a rebound from inhibition due to light which, when re-admitted, diminishes any shadow reaction that may be in progress.

We are greatly indebted to the Zoological Society of London, especially to Dr H. G. Vevers, for much assistance. We are similarly indebted to Dr D. Pye, of the Institute of Laryngology and Otology, and to Mr S. E. White of the College Science Workshop. Our thanks are also due to Miss M. E. Rablah of the Physics Department and to Drs Brindley, Denton and Pirenne, who kindly criticized the manuscript. The research was supported by a grant from the Medical Research Council.

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THE SHADOW REACTION OF *DIADEMA ANTILLARUM* PHILIPPI

II. INHIBITION BY LIGHT

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INTRODUCTION

In our previous study of the shadow reaction (Millott & Yoshida, 1960) the relation between the response and the preceding illumination and shadow led to the suggestion that the reaction may be a rebound from inhibition produced by light. Also, that the shorter duration of the reaction, together with the diminished amplitude and frequency of the contractions produced by brief shadows as compared with those produced by longer, is due to the inhibiting effect of the re-admitted light rather than to any effect of the duration of shading on processes initiated in darkness. Similarly, where the light was not cut off completely the lesser effect could be explained as due to the inhibiting action of the light remaining. A shadow would thus lack any intrinsic value as a stimulus, being a mere interruption of the inhibitory influence exercised by light.

Other explanations are possible, but a cardinal feature of this notion is the existence of an inhibitory effect of light. It is the object of this study to show that light exerts such an action.

METHODS

If light has an inhibiting effect, it should be possible to make it suppress the shadow reaction and, by arranging events so that light is re-admitted after a reaction has been set in train, the inhibition should become clearly evident.

To show that light, and not the shadow, is the factor which moulds the ensuing reaction, the shading may be kept constant and the intensity of the re-admitted light varied, as a result of which the degree of inhibition should vary in the same way.

Again, if light inhibits, it should be possible to make its effect at one point inhibit the response to a shadow cast elsewhere. Experiments based upon those reported in the previous account were designed to demonstrate this in both radial nerve and skin.

The responses of a single spine were examined in preparations of the same kind as those previously employed. The experimental tank and the means of recording spine movement, together with the signals showing the onset of light and shade, the time trace, etc., were the same as before.

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To produce timed shadows and light spots in rapid succession, in different places, two light beams were used, obtained from twin lamps in the manner already described, each focused to form a separate light spot. The optical axes were inclined so as to allow the two spots to be superimposed as required and the size and intensity of each was controlled by interposed diaphragms and filters. To facilitate manipulation, the terminal lens was a long focal distance objective, and the lamps with their ancillary lenses, etc., were moved by rack and pinion. Vertically the two moved together, but only one could be moved horizontally, and in a restricted way, to keep it parfocal with its partner. The linear horizontal distance between the light spots was measured by a scale fixed to the moving member.

Projection of the spots was timed so that the first illuminated the preparation for 5 min., after which it was extinguished so as to set up the spine response. This is referred to as spot I. After a definite interval, a second light spot (spot II) was projected on to the same or a different place. Brief intervals were timed by the automatic rotating shutter already described, which was interposed in both beams, its form being so designed as to cut off the beam from one lamp and, after a pre-determined interval, to admit light from the other. This interval was most commonly 38 msec., known from the previous study to be adequate to release a response. To avoid the disturbing effect of flicker, the lamps were operated on d.c. while the shutter was in use. Longer intervals were achieved by operating the shutter manually, the timing being only approximate; the precise timing was recorded on the photographic record and measured on the accompanying time trace.

By means of the records responses could be compared as regards reaction time (latency, as defined in the previous account), amplitude and frequency of the contractions, together with the duration of the reaction, the validity of which as a basis for comparison has already been shown (Millott & Yoshida, 1960). Responses modified by spot II were also compared with unmodified control responses, elicited by using spot I alone.

Because the responses of a particular preparation decline steadily in vigour during the day, frequent controls are necessary to reveal the extent of such decline. As an added check experiments were repeated throughout the day with light spots in the same positions, either alone or with both in sequence. Testing the responsiveness in this way also served to check that the light spots were still projected on to the radial nerve.

Modification of the shadow response by light

When the reaction elicited by a brief shadow is compared with that which follows longer shading there are conspicuous differences (Fig. 1).

The most striking appear in the later part of the reaction, particularly in its duration which is clearly curtailed. Other features are affected; the amplitude of the contractions, their frequency and their uniformity of size is diminished. When the shadows are brief, i.e. less than 40 msec., the reaction time is increased. The effect on all these is graded, increasing as the shadow is made shorter and the period during which light can affect the reaction becomes longer.

That this is due to the light is shown by the effect of the complementary variable, namely intensity. If the initial (field) illumination is kept constant, but light of different intensity is re-admitted, the effect on the duration of the reaction is as shown in Fig. 2, where the time at which light was re-admitted (abscissae) is

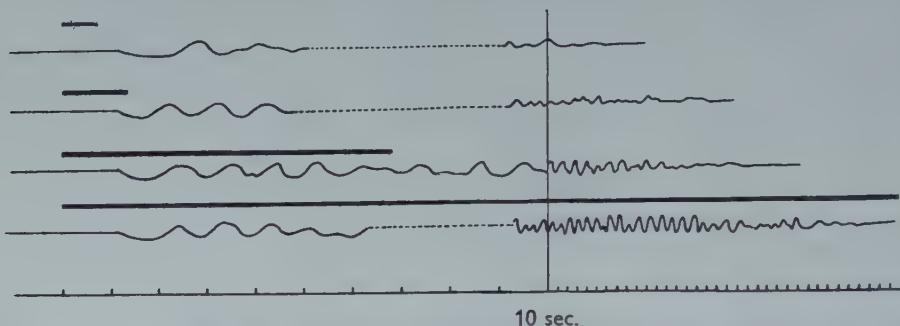


Fig. 1. The effect of prolonging shading on the character of the response. Records taken from a single preparation. The thick horizontal lines show the duration of the shadow. The breaks in the records (shown by dotted lines) are artificial and inserted to permit alignment of the later parts of each reaction. Time in seconds.

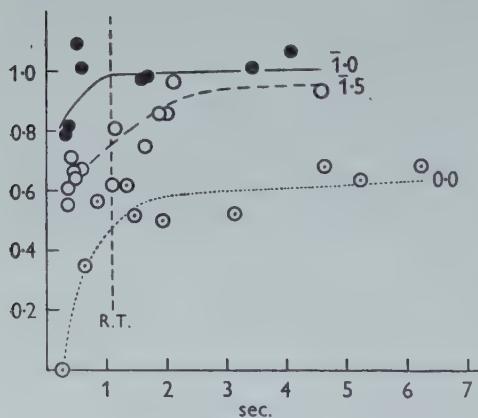


Fig. 2

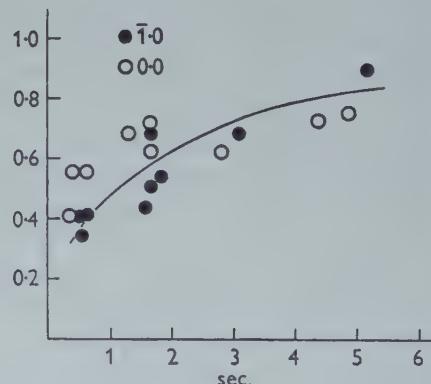


Fig. 3

Fig. 2. The effect of the intensity of light re-admitted after shading, on the duration of the response (see p. 369). Abscissae, duration of shading in seconds. Ordinates, ratio of the duration of the response to that of a control response during which no light was re-admitted. The vertical dotted line shows the reaction time. The figures alongside each curve show the relative intensity of light re-admitted, expressed in arbitrary logarithmic units.

Fig. 3. The effect of varying together the intensity of light before and after shading. Abscissae and ordinates as in Fig. 2. Open circles, illumination at full intensity; filled circles, illumination reduced by one logarithmic unit.

plotted against the duration of the reaction (ordinates) expressed as a fraction of the duration of the longest reaction, regarded as unity. The progressively shorter reaction produced by increasing intensities displaces the curves down the ordinate axis.

It is informative to compare these results with those obtained from experiments of the same kind where the intensity of the field illumination was also varied so as to make it the same as that of the re-admitted light (Fig. 3). Here there is no such displacement; the values for duration, which increase as the re-admission of light is delayed, fall on a common curve. The increased inhibition is here counterbalanced by the action of the stronger light before the shadow.

Effect of position on inhibition

In these experiments both light spots with a constant diameter (1·0 mm.) were moved along the radial nerve both orally and aborally, over a range of up to 6·0 mm.; but since this was measured on a flat scale, the true distance was slightly greater because of the curvature of the preparation.

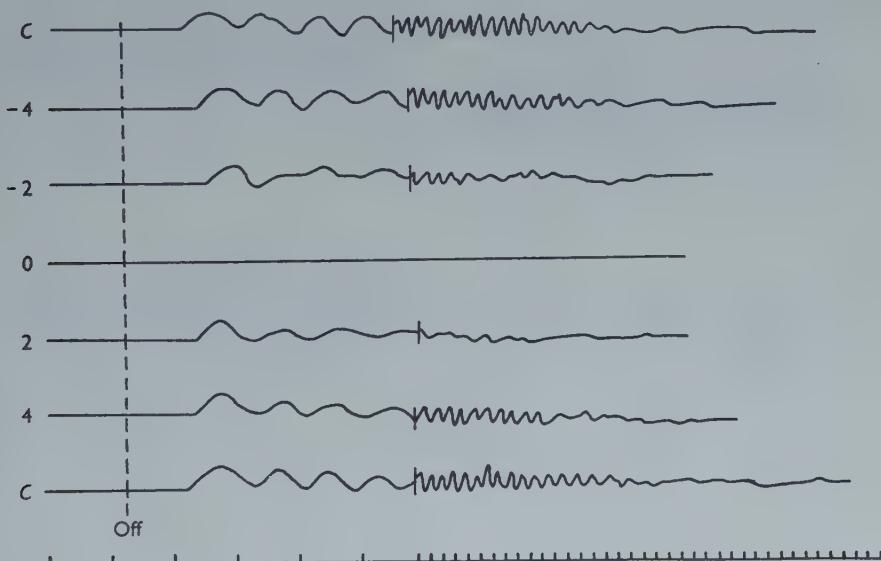


Fig. 4. The effect of the position at which light is re-admitted. Typical records taken from one preparation. The numbers alongside each record show the position of the inhibitory light spot on the radial nerve (see p. 365). C, Control reactions, during which no light was re-admitted after shading, showing the effect of extinguishing spot I at position 0, before (upper) and after (lower) taking the intervening records. Time in seconds. Vertical dotted line shows the time at which spot I was extinguished.

Typical results are shown in Fig. 4, where a standard system of notation is used; position 0 is approximately midway between the ambitus and the periproct and the degree of linear separation from this is indicated in units of 1·0 mm. on the flat scale, positive values being oral, negative aboral.

The effect of varying the position of spot II with respect to spot I is very clear. First, when the two spots are separated the inhibitory effect of spot II still appears and is exerted over distances as great as 6·0 mm. Inhibition is maximal when the two spots coincide (Fig. 4, position 0, where the reaction is completely suppressed)

or are near together, and decreases progressively as they are separated. Such a gradient appeared wherever the nerve was shaded and it affected all the aspects of the reaction we have examined, though most clearly the duration and frequency. The considerable distance over which inhibition is exerted eliminated the possibility of the effect being the result of stray light.

These effects cannot be explained as due simply to differences in sensitivity between various regions of the radial nerve, because control experiments in which spot I alone was extinguished at different points on the radial nerve revealed no comparable differences.

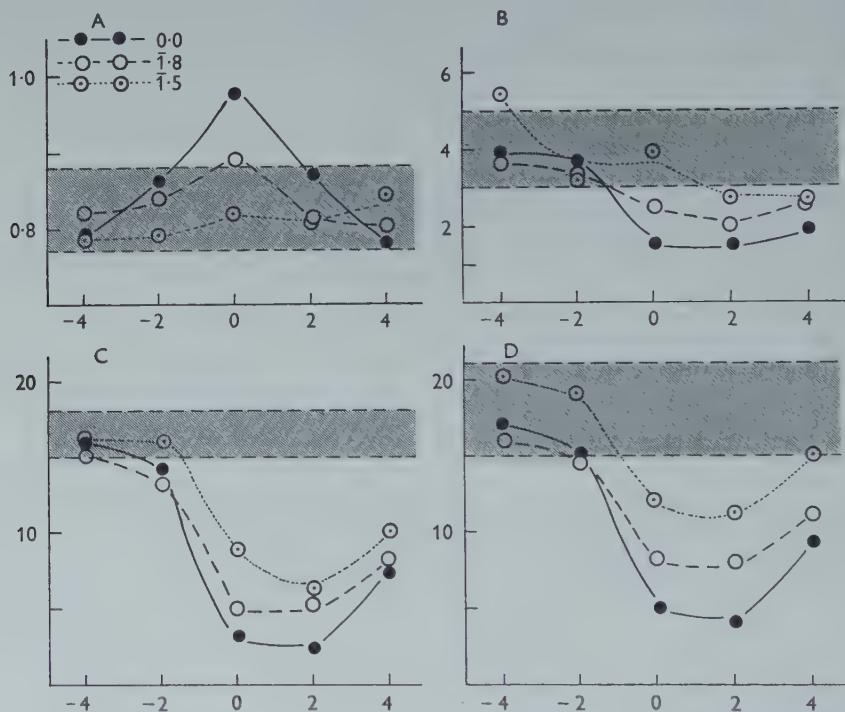


Fig. 5. The effect of increasing the intensity of spot II at various positions along the radial nerve. The results are taken from a single preparation, each point being the average of four experiments. The shaded area shows maximum range of variation in six control reactions following the extinction of spot I alone. Abscissae, in all cases, position of spot II. Ordinates: A, reaction time in seconds; B, height of first contraction in arbitrary units; C, total number of contractions recorded; D, duration of reaction in seconds. Relative intensity in arbitrary logarithmic units shown by conventions used in A.

The slope of the inhibitory gradient varied in different preparations. Most often it appeared as shown in Fig. 5, with a decidedly steeper slope when spot II is moved to the aboral side; less frequently, the decline was symmetrical and least often, the steeper slope occurred on the oral side. In some cases the effect was reversed in that, after inhibition from the second light spot had declined so as to be imperceptible, shifting it still further away resulted in a progressive increase in

the frequency of the later contractions and in the duration of the reaction after illumination. This potentiating effect was seen only in some of the instances where spot II was aboral with respect to spot I and where the inhibitory gradient had a steeper slope aborally.

Neither the position of spot I on the nerve nor the position of both light spots with respect to the spine made any difference; the same type of gradient, with its varying slopes and sometimes with potentiation, could appear in any position.

The effect of the intensity of spot II

Repetition of the type of experiment just described with variation of the intensity of the second light spot yields results of the type shown in Fig. 5.

They confirm what has been said above, the inhibiting effect of spot II increasing with its intensity. Since the shadows employed were brief an effect on the earlier part of the response (e.g. the reaction time) also appears, though it is less than that on the frequency or duration.

The results are informative in showing not only that the gradient is preserved but also that its form is reproduced at each intensity, so that the curves showing the effect of varying position are by and large shifted so as to form a parallel series (Fig. 5 C, D).

The effect of the size of spot II

This was determined by projecting spots of three sizes (0.3, 1.1 and 2.5 mm. in diameter) on to the radial nerve at five positions with respect to spot I, which was kept constant in size (1.1 mm. in diameter).

In each position inhibition increased as the area illuminated by spot II was increased. Here the effect appeared on the earlier as well as on the later parts of a reaction, so that the reaction time lengthened and the size of the initial (as well as of the later) contractions, their frequency and the duration of the reaction were reduced.

However, the size of the area illuminated by spot II affects the gradient of inhibition. With small and medium-sized spots it is of the type already described. With large spots, though the degree of inhibition is greater, the gradient is abolished and the reactions are always feeble, short and have a suspiciously long reaction time.

The explanation may be found in the controls (E 60 and 62) where projection of spot II by itself produces an 'on' response of the type already described (Millott & Yoshida, 1959), being brief and feeble with a long reaction time. This suggests that the responses following illumination of large areas may be 'on' responses and so it is necessary to be cautious in interpreting the effect of area.

The remarkably long reaction time raises a point of interest, for it is longer than when a reaction is elicited by the light of spot II alone; this means that the 'on' response has been affected, perhaps by the preceding shadow response.

Table 1. *The effect of area of spot II projected at the positions shown in the second column on its inhibiting effect (see p. 381)*

(In all cases spot I is at position 0. The initial amplitude is the size of the first contraction expressed in arbitrary units. The later amplitude is that of the largest contraction occurring 10 sec. after the beginning of shading. Very small contractions are shown by +. The number of beats is recorded in each 10 sec. interval shown.)

Size (mm.)	Spot II	Expt.	Reaction time (sec.)	Amplitude		Number of beats								Duration (sec.)
				Initial	Later	0	10	20	30	40	50	60	70	
0.3	-4	E-7	1.03	8	10	10	12	10	9	9	8	3	.	.
	-2	E-16	0.91	8	6	10	9	10	3
	0	E-4	0.97	5	3	9	7	3
	2	E-13	1.12	7	4	8	7	4
	4	E-10	0.97	5	6	12	8	6	6	4	1	.	.	.
1.1	-4	E-19	0.97	7	8	10	9	11	6	7	6	2	.	.
	-2	E-17	1.09	7	6	7	9	6	4	5
	0	E-21
	2	E-12	1.38	3	+	6	3
	4	E-11	1.15	4	1	8	6	3
2.5	-4	E-8	2.18	+	.	1
		E-30
		*E-62	1.47	1	.	1
	-2	E-15	1.97	+	.	3
		E-31
	0	E-3
		E-32	1.94	+	.	1
		*E-60	1.41	1	.	1
	2	E-14	1.91	+	.	1
		E-33	1.97	+	.	1
Controls	4	E-9	2.25	+	.	1
		E-34	2.00	+	.	1
		E-1	1.03	8	4	10	9	8	5	2
		E-18	1.03	6	7	10	7	8	8
		E-29	1.06	4	3	11	10	5	6	2

* In E-60 and E-62, spot II alone was projected, so that the response is of the 'on' type (see p. 381).

The inhibitory pathways

Information concerning the pathways of the inhibitory influence and the site of its interplay with the shadow reflex would clearly be useful. It was obtained from simple nerve-cutting experiments. Preparations of the same type were used and the experiments were repeated using light spots 0.8–1.0 mm. in diameter, after making certain cuts in the radial nerve and its branches. Several difficulties were experienced. The branch nerves are translucent and because of their looseness are sometimes difficult to cut successfully. Cuts were made by fine needles or by glass knives, under a low-power binocular microscope, before the preparation was used. After use, it was fixed and the position and completeness of the cuts were checked again and their distances from the light spots, spine, etc., were measured. Where there was doubt, the experimental results were rejected.

The disposition of the cuts is shown in Fig. 6.

Cutting across the radial nerve between the position of the two light spots (Fig. 6B) does not abolish inhibition. Thus spot II may be sited in positions corresponding to either 2 or 4 in Fig. 6B when spot I is in a position corresponding to 3.

This means that some, at least, of the interaction must occur outside the central nervous system. Such was the case with light spots as far apart as 4 mm.

On the other hand, cutting the lateral branches at positions *c* and *d* in Fig. 6C, so as to remove the direct pathways to the periphery from portions of the radial nerve as long as 3·5 mm., left the inhibitory gradients in that area unimpaired. Increasing the distance between the two light spots projected on to this region still reduced the degree of inhibition, a fact which holds, whatever the position of the spine with respect to the denervated area. This suggests interaction within the radial nerve, rather than at the effector; the interaction would be mediated by distinct peripheral pathways of excitation and inhibition, since the mechanism responsible for the gradient is preserved.

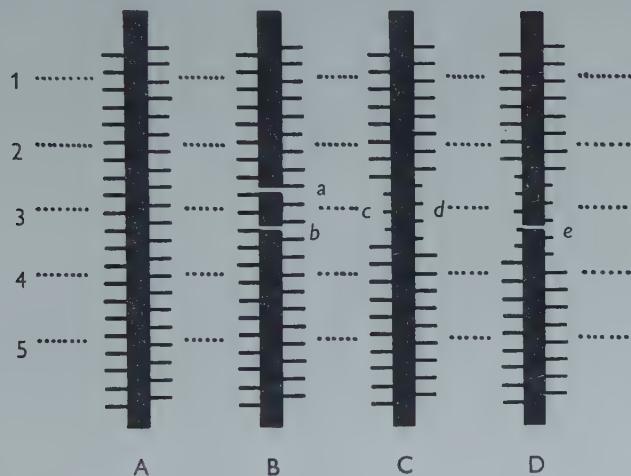


Fig. 6. Diagrams of a portion of the radial nerve and its side branches showing the size and disposition of cuts made to demonstrate the inhibitory pathways (see p. 383). Numbers mark the relative positions of the light spots projected. For letters see text.

Experiments in which the branch nerves on either side of the radial nerve for a distance of 3·5 mm., as well as the radial nerve itself, have been cut across (Fig. 6D) show that shading the radial nerve at position 3, just aboral to the cut across the main nerve (*e*), still elicits a response. This means that nerve pathways for the shading reflex can travel along about 3·5 mm. of the nerve cord before emerging to the periphery.

Similarly, projecting light spots between positions 2 and 3 (see Fig. 6D) in the same type of preparation shows that they can interact when 2·5 mm. apart. Further, since inhibition can be elicited by a spot just aboral to *e*, with the spine at either position 2 or 4, the inhibiting pathways traverse the radial nerve for at least this distance.

The effects on the reaction time are particularly interesting (Table 2). Sectioning the lateral branches (Fig. 6C) increases the reaction time of the response which follows shading the cord in the same region (pieces III and IV, Table 2); moreover,

the effect is greater if the shadow falls in the middle of the denervated area than at the ends, and is greater still if the radial nerve is then cut across, particularly when the shaded area is nearer the cut end (Fig. 6D; piece IV, Table 2). Although possible effects of denervation on the speed or responsiveness of the effector should not be overlooked, it is difficult to avoid the suggestion that the nerve cutting brings into action pathways that are more devious than usual.

Table 2. *The effect of cutting across the radial nerve and its branches on the reaction time (see p. 384).*

(The letters indicate the state of the nerve, which corresponds with that similarly lettered in Fig. 6. The position of spot I is shown by numbers corresponding to those of Fig. 6. The reaction times (in seconds) shown in each case are averages of three to five readings, except in piece I for which the average is of thirteen readings (cited from Millott & Yoshida, 1960).)

Piece	I	II		III		IV	
Operation (see Fig. 6)	A	A	B	C	D	C	D
Position of spot I (see Fig. 6)	I 1	1.26 ± 0.08
	2	.	0.78	0.94	1.34	.	1.21
	3	1.33 ± 0.05	0.81	0.97	1.51	1.50	1.87
	4	.	0.78	0.87	1.47	.	1.38
	5	1.32 ± 0.06	2.72

Another effect of transecting the radial nerve and its branches is to make the shadow response more readily inhibited. Thus although the response to extinction of one spot projected on to the transected nerve cord (e.g. at position 3 in Fig. 6B), may remain the same, the inhibitory effect of a second spot projected on to a region of the cord, separated from the first by a cut (e.g. at either position 2 or 4 in Fig. 6B), is increased, so that the reaction time is sometimes strikingly greater and the response diminished to a greater degree by light spots of the usual intensity.

The pattern of inhibition is sometimes altered by cutting across the radial nerve, so that light spots projected near to the transected region of the nerve exert a marked inhibition, whereas before cutting they produce only a slight increase in reaction time. Again, the inhibitory gradient may be altered so that the point of maximum inhibition shifts from the point where the two light spots coincide to a neighbouring position.

Finally, transection sometimes augments the later part of the response which follows the extinction of a single light spot (Fig. 7), so that the amplitude, frequency and regularity of the beats is increased, as is also the duration of the reaction. This suggests the existence of a tonic inhibitory effect in the nerve cord.

In all, there is bewildering variety of effects, indicating a complex pattern of nervous interaction that is altered by transection. Numerous alternative pathways for the response and its inhibition seem to exist and nervous interaction can occur both centrally and peripherally. To what extent these alternative pathways play a part in normal responses remains unknown.

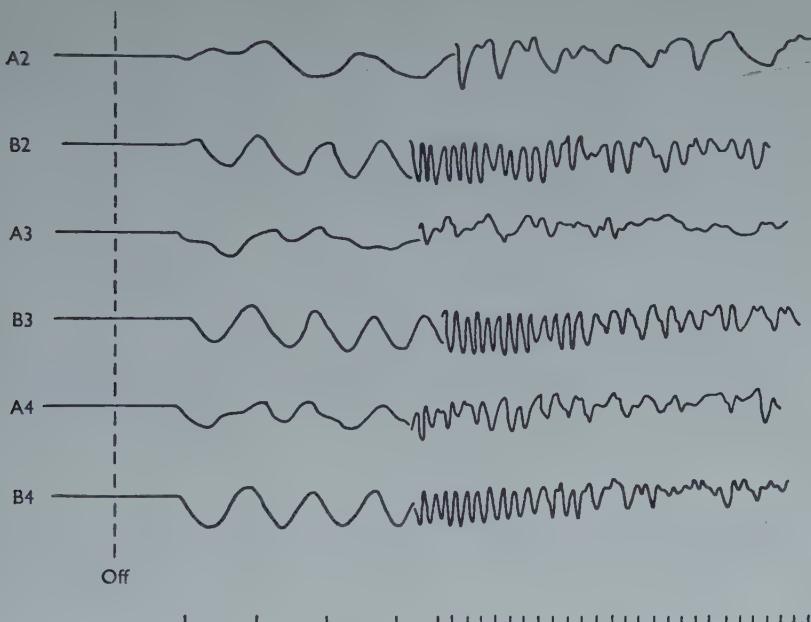


Fig. 7. The appearance of a tonic inhibitory effect in the radial nerve. Tracings marked A followed by a number result from the extinction of spot I at positions indicated by the corresponding numbers in Fig. 6. Tracings marked B followed by a number indicate the effect of extinguishing similar spots, at the positions indicated by the corresponding numbers, after cuts have been made (see Fig. 6B). In each case only a portion of the reaction is shown. Time in seconds. The vertical dotted line shows the time at which spot I was extinguished.

Inhibition and interaction at the outside surface

Preparations exactly the same as those previously described were used and the same technique was employed, except that here the preparation was held with the spine directed upwards, the two light spots being projected on to the outside (upper) surface.

Typical results from preparations reproduced in Fig. 8 show that the response to shading is inhibited by light spots projected on to most areas of the test, both ambulacrals and interambulacrals.

The inhibition is manifest in the same way as when the light spots were projected on to the radial nerves. Moreover, it forms meridional gradients, maximum inhibition being produced when the two spots fall on the same position, the effect declining as one spot is moved in the direction of the oral or aboral pole. The region over which the two spots interact may extend along a meridian for slightly more than 6 mm.

The slope of the gradient varies as in the radial nerve, not only in different preparations but also according to whether spot I is projected on to positions oral or aboral to the ambitus. It may be steeper orally or aborally and there is no consistency in the variation. Here it is pertinent to recall the irregularity of the

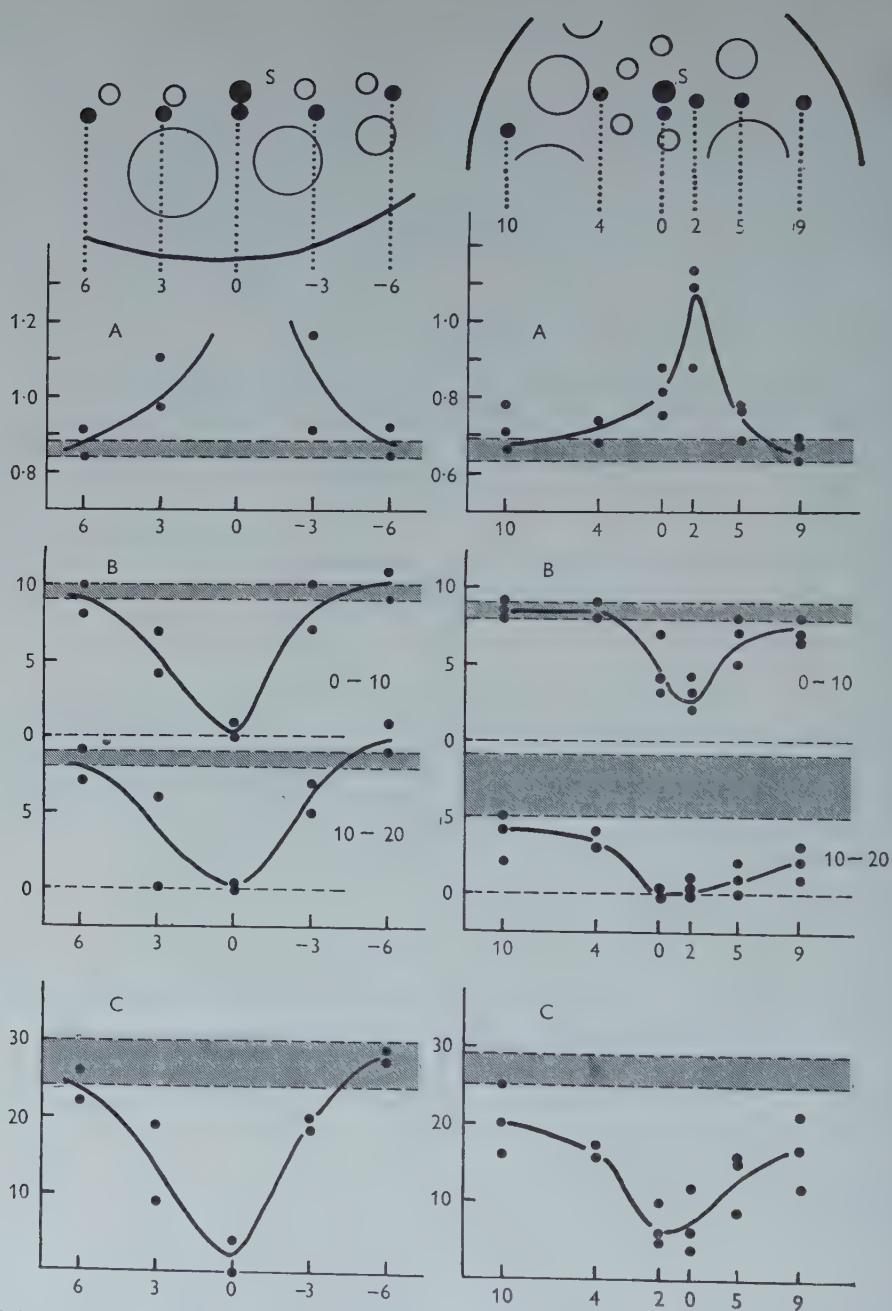


Fig. 8. The inhibiting gradient at the outside surface: on the left in a meridian, on the right in a plane parallel to the ambitus. The position of spot II is represented by the filled circles, spot I being at 0 in each case. The numerals corresponding to each position indicate the linear separation in mm. from spot I. The open circles show the position and size of the spine bases, corresponding to spines which have been removed. The position of the spine whose movements were recorded, is shown by S. Abscissae, position of spot II (the numbers corresponding with those in the diagrams at the top). Ordinates: A, reaction time in seconds (values for position 0 fall beyond the scale); B, number of beats recorded in the two successive periods shown alongside each curve; C, duration of reaction in seconds. Each vertical column of curves represents the results of one experiment on the same preparation. Shaded areas show the maximum range of variation in 6 (left) and 8 (right) control experiments where spot I alone was used.

outside surface due to the many spine bases, etc., which may to some extent account for the inconsistency.

Similar inhibitory gradients appear running parallel to the equator and these are particularly interesting since they extend over ambulacra and interambulacra, but the sensitivity, unlike that of the meridional gradients and radial nerves, is not uniform. Thus when the inhibitory effect of light spots projected on to these areas is determined and compared, it is seen that they form a gradient as follows:

ambulacral margin → ambulacral centre → interambulacrum.

This corresponds exactly with that of the reaction time (Millott & Yoshida, 1960) and the sensitivity to shading, previously reported (Millott, 1954); indeed the ambulacral margins are so clearly the most sensitive that, even though the two light spots may coincide at the ambulacral centre, inhibition is still less than when spot II falls at the margin.

DISCUSSION

The present study shows clearly that light inhibits the spine reflex and strengthens the suggestion that the shadow reaction may be the result of release from inhibition.

The other suggestion, that the effect of the shadow intensity was really that of intensity of light remaining, is also substantiated by the experiments in which light readmitted after shading is shown to inhibit the reaction with an efficacy roughly proportional to its intensity. There are thus good grounds for believing that any light remaining after shading would continue to inhibit the reaction that ensues, in the ways that the experiments described above have revealed.

The use of separate light spots has revealed more of the inhibitory mechanism involved. The effect of light spots of differing size shows that spatial summation of inhibition can occur over considerable areas. Interaction has been shown to occur, so that the shadow reaction is moulded by events in separated receptive regions, both in the skin and central nervous system.

The interaction can be complex and a variety of inhibitory patterns, some of which proved unpredictable, have appeared. There are also indications that interplay with the 'on' response may sometimes be involved. The work may well have been sufficient to reveal only a little of the complexity, and much more is required before a beginning can be made towards resolving it. Thus we have as yet paid no attention to the possible effects of sensory adaptation which affect interaction in the vision of vertebrates (Barlow, Fitzhugh & Kuffler, 1957).

The importance of nervous interplay in vision involving complex photoreceptors has been emphasized by Granit (1933, 1955) in the case of vertebrates, and by Hartline in invertebrates. Its importance in the dermal light sense of echinoids has now been revealed and it is pertinent to recall the earlier experiments on shadow reactions in *Balanus* performed by von Buddenbrock (1930), who realized the importance of illumination and showed the inhibitory effect of light, postulating the existence of interaction at nerve centres.

Clear evidence of spatial and temporal interaction in *Diadema* enables us to introduce the 'receptor field' concept into the dermal light sense. We regard the

field as the area over which interaction can occur. Although our experiments have been inadequate to reveal the pattern or even the extent of such areas, the existence of gradients showing a gradual change from inhibiting to potentiating effects recalls the complex retinal organization revealed in vertebrates by Kuffler (1953), Barlow *et al.* (1957) and Wiesel & Brown (1958). Thus in some respects the dermal light sense of *Diadema* shows a complexity which parallels that of elaborate photoreceptors. The dermal light sense may thus prove to be of much more complex organization than is commonly suspected.

There is a continually emerging parallel between the shadow response in *Diadema* and the 'off' effect in certain elaborate photoreceptors. Though the planes of analysis differ greatly, the present study extends this parallel. Responses to changes in light intensity are regarded as both prominent (Hartline, 1938a) and distinctive (Ratliff & Mueller, 1957) features of the vertebrate eye, though 'off' effects have been described in the eye of *Pecten* (Hartline, 1938b). Such responses are widely believed to be the result of interplay between excitation and inhibition in ganglionic layers associated with complex eyes. Inhibition has now been shown to play its part in the shadow reaction of *Diadema* in which the receptive surface is extensive and diffuse.

It is noteworthy that in the field (or gradient) the inhibitory effect is greatest between regions that are closest together, and this could have an effect, similar to that mentioned by Hartline (1958) in connexion with complex photoreceptors, of emphasizing the contrast generated by patterns of light and shade falling on the skin of *Diadema*. This would be particularly effective if the pattern were continually changed by a moving shadow. Such an organization would signal very effectively the position of a moving (and possibly harmful) object in the vicinity, calling forth an effective response in the poisonous spines.

SUMMARY

1. Light is shown to suppress the shadow reactions of *Diadema* spines to a degree which varies with its intensity.
2. Inhibition can occur when there is spatial separation between the areas shaded and illuminated in the radial nerve and skin.
3. The degree of inhibition is affected by the position and size of the area lighted. In both skin and radial nerves, uniform meridional gradients of inhibition are found, inhibition being maximal when the areas lighted and shaded are near or coincide, decreasing as these areas are moved apart. The effect of light may be reversed when it is projected at more than a critical distance from the shadow. Gradients in the skin which run parallel to the ambitus, show maximal inhibition at the ambulacral margins, so that the inhibitory gradient corresponds with that of sensitivity to shadows.
4. Interaction between excitation and inhibition may occur in the radial nerve or at the periphery and there are several pathways for excitation and inhibition.
5. The findings are discussed in relation to 'off' effects and receptor fields in retinae.

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THE SHADOW REACTION OF *DIADEMA ANTILLARUM* PHILIPPI

III. RE-EXAMINATION OF THE SPECTRAL SENSITIVITY

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Our previous account of the spectral sensitivity (Millott & Yoshida, 1957) was based on a study of the spine response in isolated pieces of test bearing many spines. The areas illuminated and shaded extended over the whole internal or external surface.

Recent work (Millott & Yoshida, 1960), has shown that the shadow response is not a simple reflex and that considerable interaction in the nerve pathways arising from neighbouring receptive areas occurs, so that spatial summation is involved.

Interaction between spines is now known to be significant, so that preparations with several spines behave in a slightly different way from those with only one (Millott & Yoshida, unpublished). Neither of these factors was considered in the preceding study.

The method previously employed had other defects. Thus it was difficult to ensure that the spectral quality of the light transmitted by the interference filters mounted in the apparatus used was identical with that measured when they were mounted in the spectrophotometer. Again, such factors as chromatic aberration, differential absorption, etc., in the lens system used were not taken into account. These factors have been eliminated by the method described below.

METHODS

The general principle of the experiments is to subject preparations to a fall in light intensity by changing instantaneously from white to coloured light, the intensity of which was adjusted by neutral filters so as to be less than that of the white (the intensity of which is kept constant) by an amount just adequate to elicit a shadow response.

Since the most effective wavelengths approach most closely the effect of white light, it will be with these that the most dense neutral filters are required. Less effective colours will require proportionately less adjustment. Thus the relative effectiveness of light of different colours is determined by reference to white light of a constant intensity.

Apparatus

The type of preparation, the method of mounting and the experimental aquarium were the same as previously employed (Millott & Yoshida, 1960). Movements of

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the single spine were observed on a ground glass screen alongside the shadow of a stationary 'hair', to assist detection of small movements.

The optical system is shown in Fig. 1. Two beams Op. 1 and Op. 2, produced from the tungsten filament lamps S_1 and S_2 , were focused by L_3 and L_{11} to form identical spots (0.5 mm. in diameter) at the same position on the radial nerve (N), by the means already described (Millott & Yoshida, 1960). Op. 2 was coloured by passing it through one of the same nine Balzer filters (I.F.) previously used and described (Millott & Yoshida, 1957), which pass wave bands 8–11 m μ wide at 50% transmission.

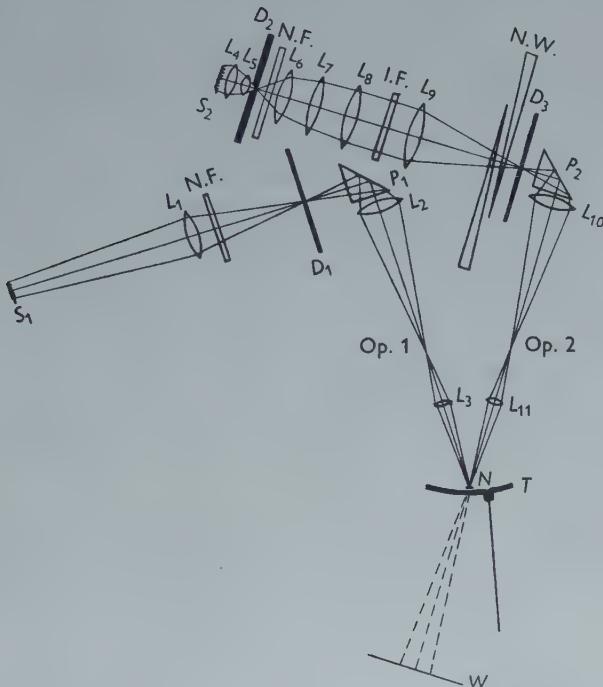


Fig. 1. Diagram of the optical system used (for explanation see text).

Instantaneous (12 msec.) change-over between the beams was achieved by a spring loaded shutter, which admitted one and simultaneously interrupted the other.

The parallel beam necessary for proper operation of the filter was obtained by the lenses L_4 to L_8 . L_4 and L_5 (a microscope substage condenser), were placed as close as possible to S_2 , so that its reduced image was formed in the plane of the diaphragm D_2 with little loss of intensity. L_6 again reduced loss of light by shortening the focal length of the two succeeding lenses.

To test whether the beam was parallel, a plane mirror was inserted in place of the interference filters, to ensure that the image of S_2 , when reflected back, appeared on D_2 at the same place as the focused image, whatever the distance of the mirror from L_8 .

The lenses L_1 and L_9 focused the beams on diaphragms D_1 and D_3 ; the instantaneous shutter operated between these and prisms P_1 and P_2 .

Each beam was controlled by the neutral filters (N.F.), and in addition the coloured beam was controlled by two identical neutral wedges (N.W.), moved in opposition so as to maintain a uniform field.

S_1 , a 12 V. lamp rated at 100 W., was operated by 5·0–5·8 V. a.c. The 115 V., 100 W. lamp S_2 , calibrated by the N.P.L., was run at 89·9 V. to give a colour temperature of 2700° K. The supply to each lamp was controlled separately by means of a voltmeter and a variable resistor.

Procedure

Experiments were performed in a darkroom at 22·0–23·5° C. In any one series of experiments the temperature did not fluctuate by more than 0·5° C.

Each piece of test (T), with its spine, was left to recover and adapt in the dark for 45–60 min., after which the spot of white light was projected on to the radial nerve for 5 min. and then replaced by the coloured. If the reaction was other than threshold the sequence was repeated after making appropriate adjustments to the neutral filters. When a threshold reaction occurred (and with experience this could be obtained quickly) the experiment was repeated several times using the same relative intensities as well as those about 0·1 log units above them, to ensure consistency. By repeating this procedure for each colour filter and comparing its effectiveness with that of the constant intensity of white light, it was possible to obtain values that could be used to calculate the relative sensitivity at each colour (see below).

The sensitivity of each preparation was checked throughout the day by determining the threshold level for the filter with maximum transmission at 465 m μ , which is known from the previous study to be near the point of maximal sensitivity.

Calculation of relative sensitivity

The relative energy (E_λ) of each colour with the same effectiveness as that of the white light is given by $\log E_\lambda = \log \eta_\lambda - D_x$, (1)

where η_λ is the relative total amount of energy falling on the receptive surface and D_x is the optical density of the neutral wedges inserted to elicit a threshold response.

The relative amount of energy (H_λ) radiated from S_2 at each wavelength (λ) can be calculated from Wien's formula, the tungsten filament being regarded as a full radiator and the lamp being run at the prescribed colour temperature. But some of the energy is dissipated by absorption in lenses, prisms, sea water, and by chromatic aberration, etc., so that the relative amount of energy (η_λ) actually reaching the preparation is given by

$$\log \eta_\lambda = \log H_\lambda - T, \quad (2)$$

where T is the transmission coefficient of the interference filter and the total absorbance, etc., of the optical system Op. 2 expressed in terms equivalent to optical density.

Table 1. Calibration of relative amount of energy

Wavelength (m μ)	Calculated relative amount of energy $= \log \eta_\lambda$	Relative amount of energy required for scotopic vision. $\log E_\lambda$ (see equation (1))			Average	Corrected relative amount of energy $(\log \eta_\lambda)$
		M.A.G.	D.B.	M.P.M.S.		
410	0.08	1.37	1.42	1.66	1.48	1.56
442	0.32	0.75	1.01	0.96	0.91	0.48
465	0.36	0.37	0.48	0.50	0.45	0.21
475	0.36	0.27	0.26	0.30	0.28	0.14
501	0.50	0.00	0.00	0.00	0.00	0.00
534	0.76	0.16	0.28	0.14	0.19	0.14
544	0.69	0.35	0.52	0.26	0.38	0.26
558	0.80	0.69	0.84	0.70	0.74	0.66
594	0.98	1.73	1.96	1.64	1.78	0.51

Table 2. Spectral sensitivity obtained by matching method

Wavelength (m μ)	E ₁			E ₂			E ₃			E ₄			E ₅			E ₆			Average		
	$\log H$	$1/H \times$	$\log H$	$1/H \times$	$\log H$	$1/H \times$	$\log H$	$1/H \times$	$\log H$	$1/H \times$	$\log H$	$1/H \times$	$\log H$	$1/H \times$	$\log H$	$1/H \times$	$\log H$	$1/H \times$	$\log H$	$1/H \times$	
410	—	—	0.86	1.38	0.96	11.0	—	—	—	0.84	14.5	0.80	15.9	0.99	1.02	0.80	1.00	0.99	0.869	13.8	0.869
442	0.04	90.9	0.01	98.0	0.02	95.2	1.99	1.02	1.98	1.04	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	0.004	98.7	0.004
465	0.00	100	0.00	100	0.00	100	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	100	0.00
475	0.16	69.0	0.16	69.0	0.21	61.7	0.21	61.7	0.20	62.9	0.21	61.7	0.21	61.7	0.21	61.7	0.193	0.193	0.193	64.3	0.193
501	—	—	0.42	38.0	0.54	28.8	—	—	—	0.38	41.7	0.38	41.7	0.38	41.7	0.38	41.7	0.436	37.6	0.436	
534	—	—	—	—	0.76	17.4	—	—	—	0.69	20.4	0.70	20.4	0.70	20.4	0.70	20.4	0.718	19.3	0.718	
544	—	—	—	—	—	—	—	—	—	0.87	13.5	0.83	14.8	0.83	14.8	0.83	14.8	0.851	14.2	0.851	
558	—	—	—	—	—	—	—	—	—	1.04	9.1	1.16	6.9	1.16	6.9	1.16	6.9	1.124	7.6	1.124	
598	—	—	—	—	—	—	—	—	—	2.08	0.8	2.08	0.8	2.08	0.8	2.08	0.8	2.255	0.6	2.255	

The magnitude of T will depend largely on the interference filters, so that any error in the previous determination of their transmission coefficients will seriously affect the sensitivity curve. Other factors may also introduce error.

A relatively simple way of determining the total error is to compare the curve obtained by using the above apparatus and equations (1) and (2) with a standard curve for the same receptor. A suitable standard is the curve for human binocular scotopic sensitivity from which relative energy values can be calculated for each wavelength.

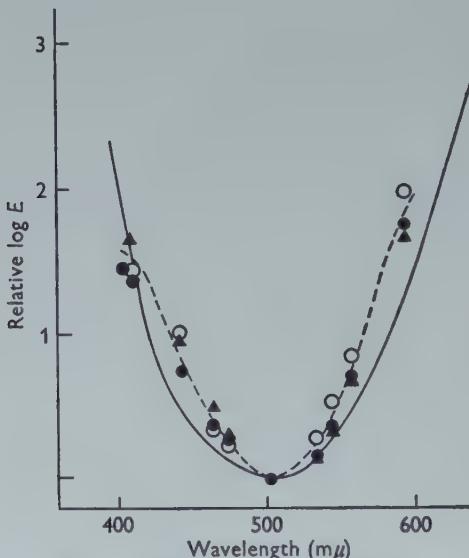


Fig. 2. Comparison of the curve for human binocular scotopic sensitivity, determined for the three subjects, ●, M.A.G.; ○, D.B.; and ▲, M.P.M.S., by using the apparatus in Fig. 1 (broken line) and a curve drawn from the standard data provided by Crawford (solid line).

For the purpose at hand, the action spectrum of the scotopic vision of three subjects, M.A.G., D.B. and M.P.M.S., was constructed by determining the relative threshold intensity of each colour after 50 min. dark adaptation. To produce the coloured light the same optical system was used as for the sea urchins, except that the light spot was projected on to white paper (W) to form circles 15 mm. in diameter 30 cm. from the observer. The curve for the average sensitivity is shown in Fig. 2 alongside the curve drawn from data given by Crawford (1949) which form a basis for the C.I.E. standard.

There is a significant difference (Table 1, Fig. 2), and therefore the values of η_λ should be corrected to the values η'_λ , which can be obtained by the equation

$$\log \eta'_\lambda = \log S_\lambda + D_x, \quad (3)$$

where S_λ is the mean relative energy value at a given wavelength calculated from Crawford's data.

These corrected values may now be substituted in equation (1) to obtain the relative effectiveness for each colour.

RESULTS

The action spectrum obtained by matching

The data obtained from six *Diadema* are summarized in Table 2 and Fig. 3. In all cases, the relative amount of light energy (passed by the filter with maximal transmission at $465 \text{ m}\mu$) required to produce a threshold response was taken as unity. In addition, Table 2 shows the relative sensitivity to this filter (expressed as a percentage). In half of the cases this light proved to be most effective, but in the others slightly greater sensitivity was found with a filter transmitting maximally at $442 \text{ m}\mu$.

This, and more particularly the shape of the curve of mean values (Fig. 3), suggest that the real maximum is somewhere between 455 and $460 \text{ m}\mu$. Unfortunately a filter with a peak transmission in this vicinity was not available, so that we are unable to define the maximum more closely.

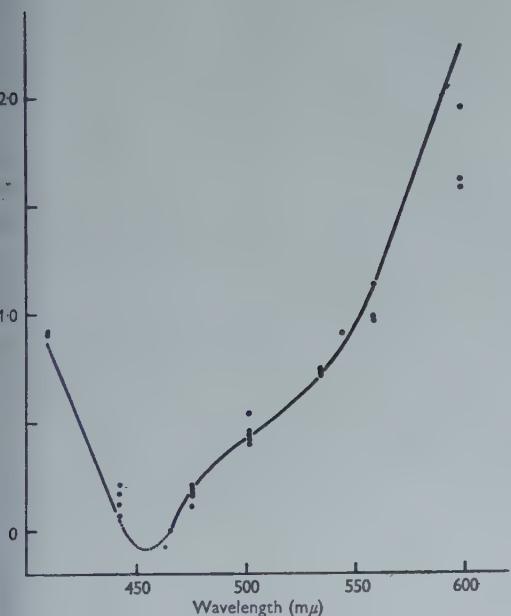


Fig. 3

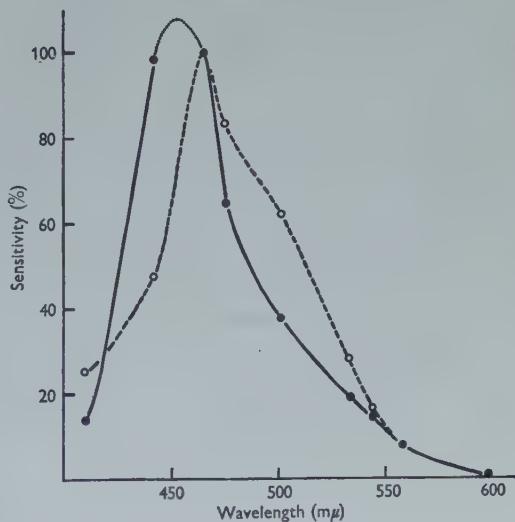


Fig. 4

Fig. 3. *Diadema antillarum*. Comparison of the average data obtained by the matching method (solid line), with the corrected data (filled circles) from Millott & Yoshida (1957).

Fig. 4. Comparison of the action spectra of *Diadema* obtained by the matching method (solid line) with that previously obtained by Millott & Yoshida (broken line).

Comparison with previous results

When compared with the results previously obtained, the present results yield a curve similar in form but with the maximum shifted some 5 to 10 $\text{m}\mu$ nearer the violet (Fig. 4).

This may be due to any of the sources of error suspected (p. 390), but if we assume that it is mainly due to the errors in the transmission coefficient previously determined for the interference filters (Millott & Yoshida, 1957), we may assess the effect by applying a correction based on the differential between the values for E used in the preceding study and those derived from Crawford's data. When this is done, the filled circles which result from the data previously obtained (Millott & Yoshida, 1957) coincide with the curve obtained by using the present matching method (Fig. 3).

Table 3. Correction of the data obtained by Millott & Yoshida (1957), see p. 396.

Wavelength (m μ)	Average relative energy (E) necessary to elicit a response	$\log E$	Corrected $\log E$
410	3.93	0.59	0.91
442	2.13	0.33	0.14
465	1.00	0.00	0.00
475	1.21	0.08	0.18
501	1.64	0.21	0.45
534	3.53	0.55	0.74
544	6.12	0.79	0.91
558	12.0	1.08	1.04
598	108.0	2.03	1.76

DISCUSSION

The action spectrum previously published for the shadow response is inadequate. The close similarity between the results now obtained by the matching method and the corrected data from the preceding account, shown in Table 3 and Fig. 3, suggests that of the sources of error originally suspected (p. 390) only those arising in the optical system are significant. No significant difference appears to arise from the stimulation of large areas and from spatial summation, which suggests that the receptive system is homogeneous in its effective absorption.

The possible significance of the abundant pigment resembling echinochrome A (Millott, 1957), discussed in the preceding paper, remains uncertain.

SUMMARY

1. The action spectrum of the shadow response of *Diadema* spines was redetermined by a matching method and a procedure which eliminated certain defects of that previously used.
2. Maximal sensitivity occurs between 455 and 460 m μ . When compared with the curve previously obtained the maximum is shifted 5–10 m μ toward the violet, but the form of the curves is similar and when the earlier curve is corrected by a factor obtained in the present study the two coincide.

We wish to thank the Zoological Society of London, especially Dr H. G. Vevers, for much help. Our thanks are also due to Dr E. Denton who suggested the method, to Dr H. J. A. Dartnall and Dr B. H. Crawford, who gave advice and criticized

the manuscript and to the Institute of Ophthalmology, for loan of the Balzer filters. We are also indebted to Mr S. E. White of the Science Workshops, Messrs M. Gross and D. Burton and to Miss M. Sargeant. We also record our gratitude to Mr Pulfer of A.E.I. for advice and the gift of a tungsten filament lamp.

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THE CUTICULAR PATTERN IN AN INSECT— THE INTERSEGMENTAL MEMBRANES

By M. LOCKE*

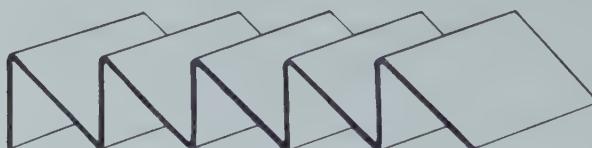
Department of Zoology, University of Cambridge

(Received 9 January 1960)

(With Plate 6)

INTRODUCTION

On the abdomen of adult *Rhodnius prolixus* there is a segmentally repeating pattern of transverse ripples. In grafting operations within a segment it has been found that the pattern is only disturbed when grafts are interchanged in the axis, showing that the cells responsible for the pattern resemble one another from side to side but differ from head to tail (Locke, 1959*a, b*). This axial difference was described as a gradient of incompatibility, for the host cells recognize the level of a graft and respond by maintaining the side-to-side continuity of the ripple pattern anterior or posterior to it. The anterior region of the segment was said to be high in the gradient because the anterior ripples join up with one another when there is competition between anterior and posterior ripples to maintain continuity. It is convenient to picture the segmentally repeating gradient as in Text-fig. 1.



Text-fig. 1. Diagram of the segmentally repeating gradient referred to in the text.

The operations which gave rise to this description were all performed within the area of integument destined to form the ripple pattern in the adult. In this way complications involving other patterns were avoided, but the results only apply to this area. It was therefore important to see if the incompatibility gradient included other patterns as part of the segmental organization as is implied in Text-fig. 1. In this connexion the intersegmental membranes are of particular interest for they separate the tail end of the gradient in one segment from the top end in the next. This problem, although one of cell behaviour, has again been studied by way of the cuticle secreted by the cells, ignoring for the moment the more fundamental problem of the relation between the cells and the cuticle pattern.

* On leave from the Department of Zoology, University College of the West Indies.

METHODS

The techniques have already been described (Locke, 1959a). Operations were performed in 4th- or 5th-instar *Rhodnius* larvae and the effects observed in the adult. The results of many experiments were confirmed by taking grafts from the sternites which have a darker pigmentation and retain the plaques in the adult.

RESULTS

(1) *The segmental nature of the gradient*

At the margins of the intersegmental membranes the ripple pattern becomes an irregular reticulum. This region was frequently included with the ripple pattern in the earlier grafting experiments in which it appeared to react in the same way, suggesting that the gradient occupies the whole area between the intersegmental membranes regardless of minor variations in pattern. It remained to be seen whether this also applied to other segments with a different type of pattern.

The nature of the ripple pattern makes it ideal for detecting displacements in the axis. It is relatively easy to interpret the way in which the ripple lines are displaced and link up in response to grafting operations. This advantage is lost when experiments are performed upon other segments, for example the thorax, where the pattern is more complicated. However, there is a simple way round the difficulty which shows at the same time the extent of compatibility between different segments. The reaction of the ripple lines can be observed in the abdominal segments when grafts of different pattern are implanted, the changes in the graft itself being ignored because of the difficulty of interpretation. The technique adopted has been to excise a square of integument from the test site and divide it transversely, implanting each rectangle in adjacent abdominal segments.

On the second abdominal tergite the ripple pattern is replaced in the midline by one of curved ridges (Pl. 6, fig. 1). Transplants were made as in Text-fig. 2. The grafts survived, and the host responded by restoring the continuity of the ripples anteriorly or posteriorly according to the origin of the graft (Pl. 6, figs. 2, 3). Thus the gradient in the 2nd abdominal segment resembles that in the 4th and 5th, although the cuticular pattern is very different. The gradient, then, is a segmental phenomenon not restricted to the ripple pattern.

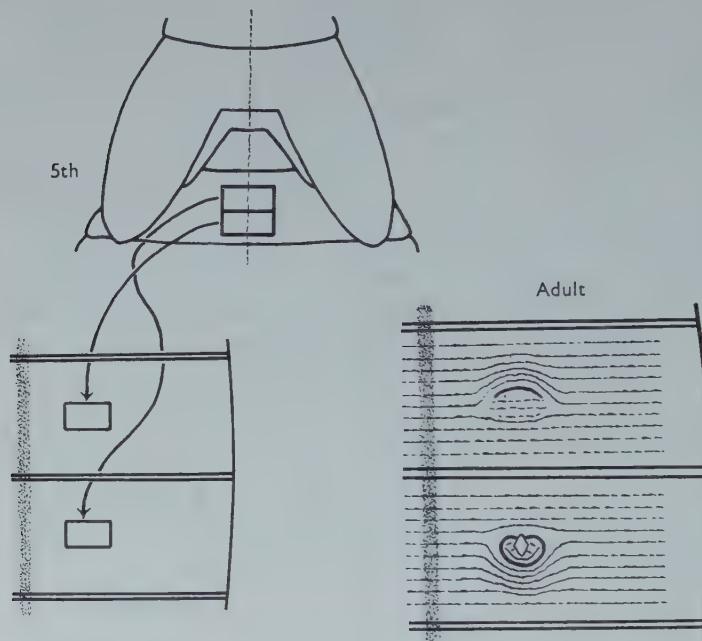
(2) *The intersegmental membranes*

The segmental nature of the gradient makes the intersegmental membranes of interest. If they behave in the same way as the ripple cuticle it could be as part of the beginning or end of the gradient.

Squares of integument with an intersegmental membrane were cut and interchanged from side to side. The intersegmental membranes always linked up with one another. Squares of integument containing the intersegmental membrane were also cut and interchanged between segments. There was no distortion in the adult even when host and graft intersegmental membranes were morphologically

distinguishable as in Pl. 6, fig. 4. Thus the intersegmental membranes are similar from side to side and in different segments, and in these ways resemble the ripple cuticle, but this is not sufficient to establish their position within the gradient.

This point has been resolved by studying the effects upon the ripple pattern of a cut edge of the intersegmental membrane. If a small square of integument with an intersegmental membrane is excised there may be complete regeneration. Thus



Text-fig. 2. The effect upon the adult ripple pattern of implanting grafts of another pattern from two different levels in a segment (cf. Pl. 6, figs. 1-3).

the intersegmental membrane can regenerate in an ordered fashion. When larger squares of integument are excised the ripple pattern is displaced into the wound so that the ripples end at the intersegmental membranes as if they were attracted there (Pl. 6, fig. 5). This effect is also noticeable on grafts. A square of integument with intersegmental membrane was rotated through 90° as in Text-fig. 3. In the adult the ripples of the graft had curved round on each side to meet the intersegmental membrane. On the host, too, ripples from each segment had united with the intersegmental membrane. From this experiment it is clear that during regeneration the intersegmental membrane does not select or reject ripple cuticle of a particular level.

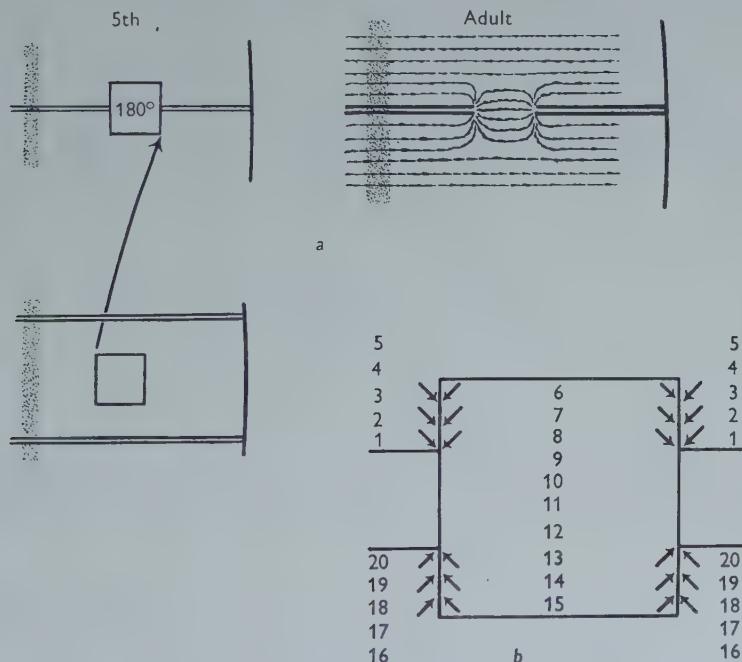
A square of integument was excised from the centre of a tergite, rotated through 180° , and implanted in a hole in the intersegmental membrane as in Text-fig. 4a. The ripples on the graft were deflected at each side to unite with the intersegmental membrane (Pl. 6, fig. 6). Thus even though the ripples are disoriented by 180°

to the host they are not rejected by the intersegmental membrane. This behaviour is quite different from the rejection of disoriented grafts within the ripple pattern.

A square of integument containing an intersegmental membrane was excised, rotated through 180° and implanted in the centre of a tergite (Text-fig. 5a). In the adult the host ripples point to the cut ends of the intersegmental membranes of the graft although they are oriented at 180° to one another (Pl. 6, fig. 7).



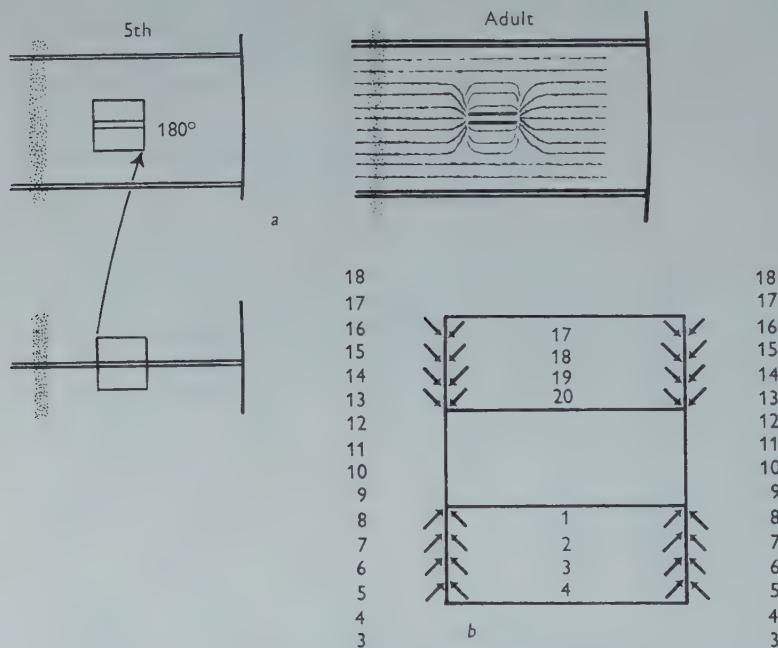
Text-fig. 3. The effect upon the adult cuticle of rotating a graft containing an intersegmental membrane through 90° .



Text-fig. 4. (a) The effect of implanting a graft from the centre of the ripple pattern into a hole cut in the intersegmental membrane after rotating it through 180° (cf. Pl. 6, fig. 6). (b) A diagram of this graft in which the gradients are represented by numbers and arrows indicate the predicted direction for the regeneration of the ripple pattern.

This movement of the cut ends of the ripples to the intersegmental membranes could be the result of a general attraction of the intersegmental membrane for the rippled ends, or it might result from the interaction of rippled gradients if the inter-

segmental membrane were neutral. A square of integument was excised from the centre of a tergite and implanted with normal orientation in a hole cut in the inter-segmental membrane (Text-fig. 6a). If the intersegmental membrane has any general attraction for free-ending ripples the pattern in the adult should be similar to that in Text-fig. 4a above. The result is shown in Pl. 6, fig. 8. The intersegmental membrane can have no such attraction, for the graft was invariably isolated in a discontinuity pattern of its own. This was occasionally double as in the upper diagram, but more often single, when sometimes the intersegmental membrane had reunited anterior to it.

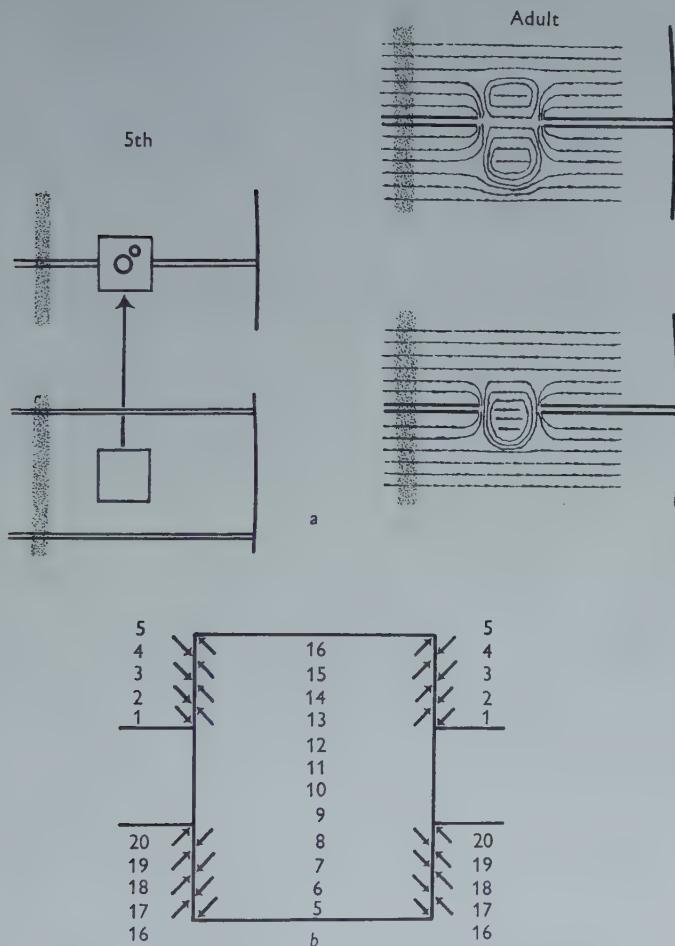


Text-fig. 5. (a) The effect of implanting a graft containing an intersegmental membrane into the centre of the ripple area after rotating it through 180° (cf. Pl. 6, fig. 7). (b) A diagram of this graft in which the gradients are represented by numbers and arrows indicate the predicted direction for the regeneration of the ripple pattern.

The results of the experiments described in Text-figs. 4a, 5a and 6a are readily understood if the intersegmental membranes are neutral with respect to the gradient behaviour. The movement of the ripples can then be ascribed to the interaction of host and graft cells, their recognition and reaction to one another's level. In Text-figs. 4b, 5b and 6b numbers have been used to indicate the gradient. The arrows indicate the direction of ripple movement expected from an interaction of the gradients. The host ripples move anterior or posterior to the graft relative to the whole animal. The distortion of the host ripples is precisely that expected from a consideration of the gradients. The graft ripples move anterior or posterior relative to the original orientation of the graft in a manner which can also be

predicted (except for the single pattern in Text-fig. 6a for which see below.) In general, when cut edges of ripples are opposite the intersegmental membranes or are forced opposite by the host-graft interaction they stay put.

From these experiments we may conclude that the intersegmental membranes are not part of the segment exhibiting the gradient behaviour.

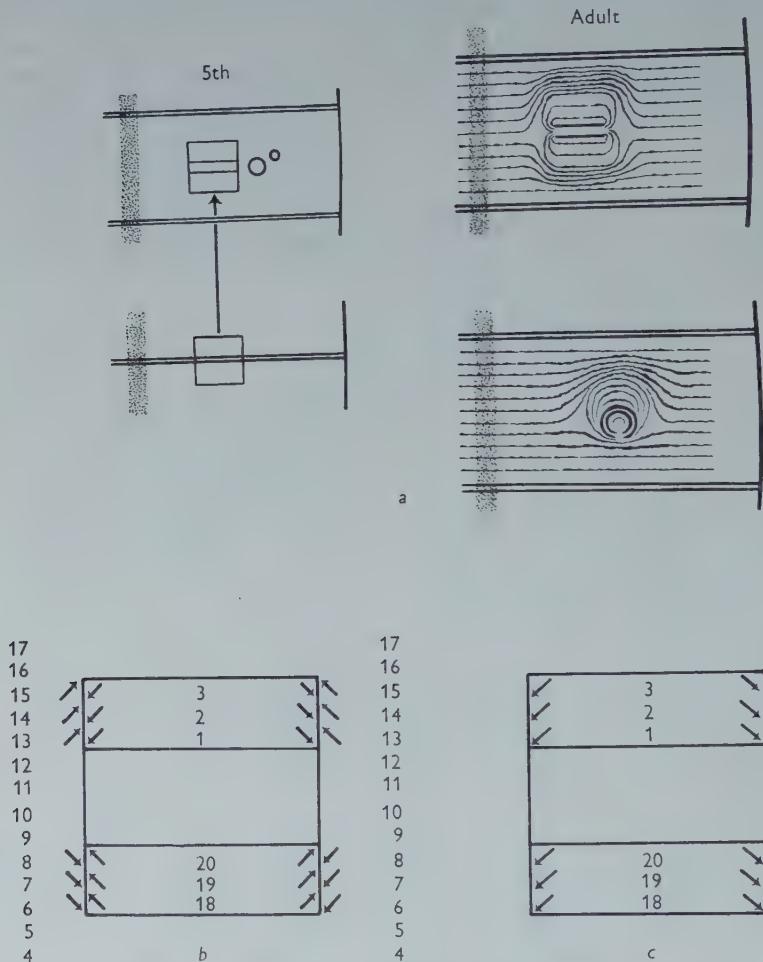


Text-fig. 6. (a) The effect of implanting a graft, cut with normal orientation from the centre of the ripple pattern, into a hole in an intersegmental membrane. The predicted pattern in the upper diagram of the adult was less common than the other (cf. Pl. 6, fig. 8). (b) A diagram of this graft in which the gradients are represented by numbers and arrows indicate the predicted direction for the regeneration of the ripple pattern.

(3) The regeneration of the intersegmental membrane

When a square of integument containing an intersegmental membrane is excised and implanted with normal orientation into the centre of a tergite, the gradients are apposed as in Text-fig. 7b. If the graft came from a tergite the double dis-

continuity pattern predicted from Text-fig. 7*b* usually resulted in the adult. But when the graft came from a sternite the pattern described in Text-fig. 7*a* and Pl. 6, fig. 9, commonly occurred. The host pattern is distorted as predicted but the graft forms a single concentric pattern. It is as if the graft intersegmental membrane had



Text-fig. 7. (a) The effect of implanting a graft containing an intersegmental membrane into the centre of the ripple area. When the graft was from a tergite the predicted pattern was usual. When the graft was from a sternite the single concentric discontinuity pattern resulted (cf. Pl. 6, fig. 9). (b) A diagram of this graft in which the gradients are represented by numbers and arrows indicate the predicted direction for the regeneration of the ripple pattern. (c) A diagram showing the gradients in an isolated graft and the direction expected for regeneration uninfluenced by the host.

its own powers of regeneration to restore continuity. When the interaction is between tergite and tergite the graft is strongly influenced by the host and the predicted double pattern results. When the interaction is between tergite and sternite the host influence is weaker and the graft restores its pattern as if it were

in isolation (Text-fig. 7c). In isolation the dominance of the anterior regions of the segment in regeneration play a part in the formation of the pattern. On each side of the intersegmental membrane the anterior parts would begin to enclose the posterior. If the intersegmental membrane regenerates between them the concentric pattern may be accounted for.

Thus the pattern formed by the graft depends very much upon the influence of the host. A variable influence of the host may also account for the anomalous results given by some preparations described in Text-fig. 6. From the apposition of host and graft gradients described in Text-fig. 6b the graft would be expected to form a double discontinuity pattern, but more frequently a single concentric pattern developed. The single concentric pattern has the form expected for regeneration in isolation, the anterior ripples uniting round the posterior part of the graft. This could be interpreted to mean that when the host ripples have migrated in to end at the intersegmental membrane they cease to influence the graft.

The capacity of the intersegmental membrane for regeneration may be satisfied in one of two ways. It may join up with itself following the track laid down for it on either side by the segmental pattern, itself kept in place by the gradient behaviour (this occurs after burns or excisions and in the isolated graft described in Text-fig. 7a). Or it may join up with the ripple pattern at any level in the segment as long as the ripples are not directed away by the gradient on each side of the intersegmental membrane.

DISCUSSION

The first impression gained from grafts involving intersegmental membranes is one of confusion because the regeneration in the graft does not always seem to follow the pattern expected from the gradient concept. The host reaction is consistent, and provides a valuable confirmation of the gradient description, but the regeneration in many grafts behaves as if it is but little influenced by the host. The effect is analogous with the single discontinuity pattern resulting when a graft of ripple cuticle is rotated through 180° (Locke, 1959a). Here also a double pattern is expected but only occurs in large grafts. This might be explained if the host only exerts its effect on the graft while its own pattern is being re-ordered. Once this is completed the graft is left with only its own orientation as a guide for regeneration. This interpretation is confirmed by many details. Small grafts, in which host repair would be quickest, most commonly result in single discontinuity patterns. In a graft from an anterior region implanted posteriorly, the host should influence the graft ripples to regenerate in the anterior direction, that is, opposite to the direction of regeneration in isolation. The result is frequently intermediate (Locke, 1959a, text-fig. 8; pl. 9, fig. 14). Tergites influence grafts from tergites more strongly than grafts from sternites. Bearing this interpretation of the apparent anomalies in mind, the gradient concept is still useful in interpreting the behaviour of grafts involving the intersegmental membranes.

Grafts from the non-ripple pattern on the 2nd abdominal segment react with the

host in the same way as grafts from other abdominal segments, showing that the gradient is not restricted to the areas with a ripple pattern. But this does not mean that all integument is ordered on this common plan. Indeed it has been found that grafts from the thorax and from the sides of the segment are consistently and completely suppressed. Grafts may be of three sorts, those in which the regeneration pattern is influenced by the host, those in which the pattern regenerates solely in relation to the graft, and those which although apparently healthy are completely suppressed by the host in one moult.

SUMMARY

1. Grafts including intersegmental membrane have confirmed the existence of a segmentally repeating gradient of incompatibility in the integument of *Rhodnius*.
2. When host and graft influence one another the pattern can be predicted from the interaction of the gradients. When a graft is uninfluenced by the host the anterior part of the graft is dominant in restoring pattern continuity.
3. The intersegmental membranes are neutral with respect to the gradient behaviour.
4. The gradient is not restricted to the area forming the ripple pattern but includes other patterns in the abdomen.

I am grateful to Prof. V. B. Wigglesworth for the hospitality of his department during my 6 months leave.

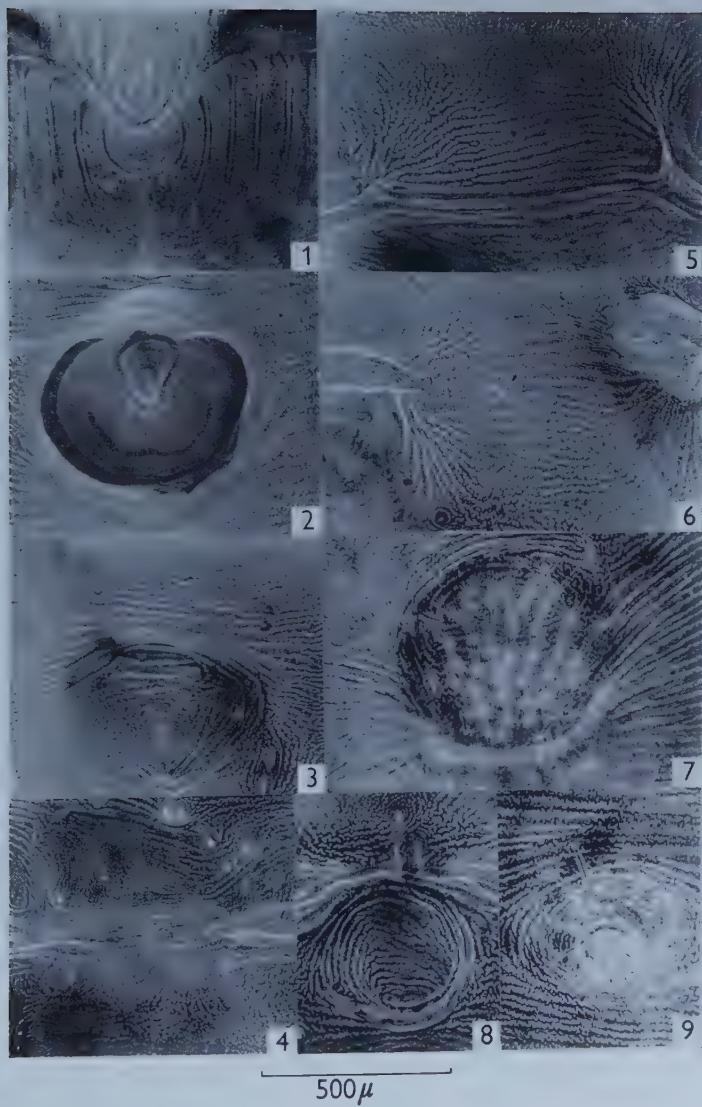
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EXPLANATION OF PLATE 6

All figures are unstained whole mounts of the abdominal tergites of adult *Rhodnius* taken with a phase-contrast microscope and oriented so that the head end is at the top of each figure.

Fig. 1. Abdominal segment 2 and part of abdominal segment 1 in the midline.
 Fig. 2. The effect upon the ripple pattern of implanting a graft from the anterior half of segment 2 (cf. Text-fig. 2).
 Fig. 3. The effect upon the ripple pattern of implanting a graft from the posterior half of segment 2 (cf. Text-fig. 2).
 Fig. 4. The intersegmental membrane from segments 2-3 can be grafted to replace that between segments 4-5 although it has a different structure.
 Fig. 5. Partial regeneration of an intersegmental membrane which had been burned in the 5th instar.
 Fig. 6. A graft from the centre of the ripple pattern has been rotated through 180° and placed in a hole in the intersegmental membrane (cf. Text-fig. 4).
 Fig. 7. A graft with an intersegmental membrane from a sternite has been rotated through 180° and placed in a hole in the centre of the ripple pattern (cf. Text-fig. 5).
 Fig. 8. A graft from the centre of the ripple pattern has been placed in a hole in an intersegmental membrane with normal orientation. In the preparation illustrated the intersegmental membrane has begun to regenerate anterior to the single concentric discontinuity pattern (cf. Text-fig. 6).
 Fig. 9. A graft with an intersegmental membrane from a sternite has been implanted in the centre of a tergite with normal orientation (cf. Text-fig. 7).



M. LOCKE—THE CUTICULAR PATTERN IN AN INSECT—THE INTER-SEGMENTAL MEMBRANES

(Facing p. 406)

RESPONSES OF THE HELIOZOON *ACTINOPHRYX SOL* TO PREY, TO MECHANICAL STIMULATION, AND TO SOLUTIONS OF PROTEINS AND CERTAIN OTHER SUBSTANCES

By J. A. KITCHING

Department of Zoology, University of Bristol

(Received 27 January 1960)

(With Plate 7)

INTRODUCTION

The Heliozoa are carnivorous. Suitable microscopic organisms which swim onto the axopods of *Actinophrys* and *Actinosphaerium* are in some way held fast; a membranous funnel grows out and surrounds the prey; and digestion proceeds in the resulting food vacuole, which is drawn gradually into the body of the captor (Looper, 1928).

This study began with an investigation of the feeding reactions of *Actinophrys*, and in particular with the conditions which lead to the outgrowth of the membranous funnels. This led to observations on the curious phenomenon of the elevation and separation of a sheath or 'skin' from the body surface, which takes place in response to the presence of certain substances in solution in the outside medium.

MATERIAL

A clone of *Actinophrys sol* was grown on a diet of *Colpidium* and *Astasia* in Bristol tap water. The stock cultures were normally fed once a week, and were used 5–10 days after feeding.

Bovine gamma globulins fraction II, bovine plasma albumin fraction V, crystallized bovine plasma albumin, and crystallized egg albumin were obtained from the Armour Laboratories, flake egg albumin from B.D.H., and bacteriological peptone from Hopkins and Williams.

OBSERVATIONS ON FEEDING

Material

The ciliates *Tetrahymena pyriformis*, *Colpidium* sp., *Colpoda cucullus*, and the flagellates *Peranema trichophorum*, *Astasia longa*, *Chlamydomonas pulsatilla* and *Chlamydomonas globosa*, were used as test food. *Tetrahymena pyriformis* was originally received from Dr Muriel Robertson, F.R.S., and all the remainder except *Colpidium* sp. were supplied by Mr E. A. George from the Cambridge culture collection.

Methods

Usually a drop of *Actinophrys* culture and a drop of the culture to be tested as food were mounted together under a cover-glass supported by filter-paper. Sometimes the Heliozoa were mounted first and the food was added by irrigation. When *Tetrahymena pyriformis* was given as food, this ciliate was first transferred gradually to Bristol tap water since its normal culture medium had a rather high osmotic concentration.

Capture of prey

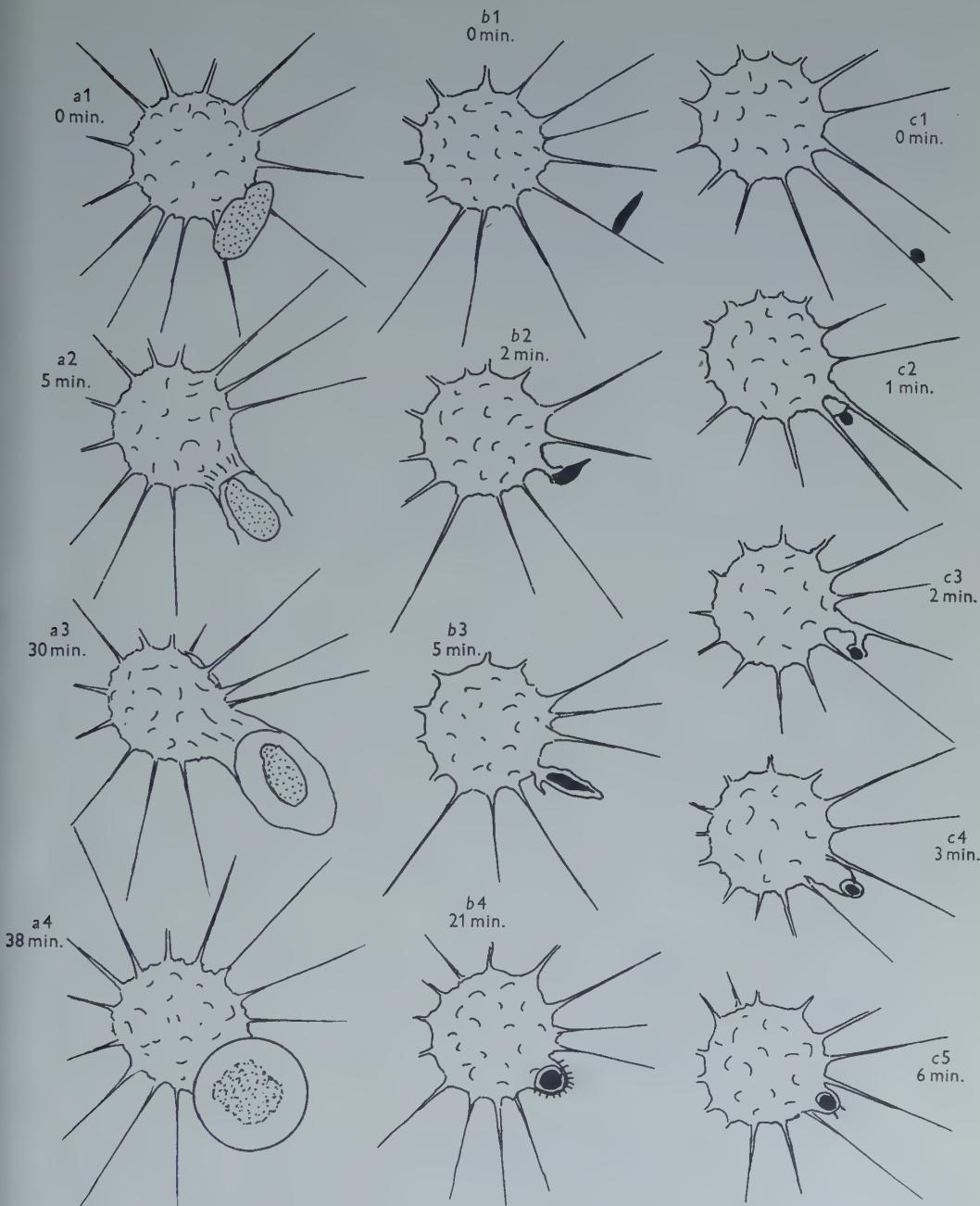
Organisms which happened to touch the axopods of *Actinophrys* were seen to stick. Sometimes they only stuck momentarily, sometimes they broke loose after further swimming movements, and sometimes they remained stuck. Flagellates usually got caught on the distal parts of axopods and gradually moved along the axopods towards the body of the *Actinophrys* until engulfed by a membranous funnel. It is not clear whether the movement towards the body was due to the prey itself or to the axopods. All the flagellates tested underwent this process except *Chlamydomonas globosa* which adhered to the axopods and usually (but not always) escaped. Small ciliates swam between the axopods and were possibly guided by these, until they hit the body surface proper. Fast-swimming ciliates often broke loose again. Flagellates normally remained stuck long enough to be enveloped by a funnel, and small ciliates also, provided they were not excited.

Swimming movements normally continued in ciliates, and often in flagellates, after they had stuck; writhing or euglenoid movements were often made by euglenoid flagellates at this stage. There was no evidence of any rapidly spreading paralysis. Adhesion was by the flagella and cilia, and it appeared that this adhesion would explain the limitation of the movements of the prey in the early stages of captivity. Flagellates and ciliates which had broken loose often had small beads or droplets on the flagella or cilia which had been held fast. Sometimes they swam away, but usually their swimming movements were abnormal, and sometimes they stopped moving.

Outgrowth of membranous funnels

Stages in the engulfment of prey are illustrated in Text-fig. 1. Outgrowth of a membranous funnel only occurred soon after the prey had touched the body surface (excluding the axopods) of the heliozoon, or the thickened base of an axopod. Thus it was often delayed for flagellates, but with ciliates it often happened very quickly after first contact of prey with predator. Outgrowth sometimes happened even if the ciliate was moving very fast and failed to stick; and sometimes a series of collisions provoked the formation of numerous unsuccessful funnels.

All the species of flagellates mentioned as test food were at some time eaten by *Actinophrys sol*. Of the ciliates, *Colpoda cucullus* was captured most readily, probably because it is a rather weak swimmer. Of the flagellates, *Chlamydomonas globosa* was only occasionally eaten; it stuck to the axopods but usually failed to move along them, so that no funnels grew out.



Text-fig. 1. Diagrams showing the capture of (a) *Tetrahymena pyriformis*, (b) *Astasia longa*, and (c) *Chlamydomonas pulsatilla*, by *Actinophrys sol*.

Food vacuoles

As soon as a funnel had closed to form a food vacuole, the prey became more active, probably because it was no longer held by an axopod. Ciliates turned round and round, and flagellates writhed. Movements of the prey continued for a period ranging from a few minutes to half an hour; and the contractile vacuole of the prey was seen to continue operating. Ultimately all activity ceased, and the surface membrane of the prey suddenly disintegrated.

Meanwhile the food vacuole was drawn closer to the body of the *Actinophrys* and became partly embedded in it. Small protuberances grew out from the outer edge of the food vacuole.

POKES AND SQUIRTS

General methods

Actinophrys was mounted in a hanging drop of its own culture medium, sealed from evaporation with mineral oil (Boots's medicinal paraffin BP). A de Fonbrune micromanipulator and microforge were used. Pipettes normally had an internal diameter of $1\text{--}2\mu$. Test solutions were made in Bristol tap water.

Results

If a glass probe or a micropipette filled with tap water was placed with the tip in contact with the body surface or with an axopod base, it was usually held immediately as though stuck. Within a minute or two a small cup grew out around it, investing it closely (Text-fig. 3). The cup then withdrew, and the instrument was usually released within 10–20 min. Sometimes a vacuole was formed by closure of the cytoplasmic cup, and this was absorbed into the body.

In many cases if an axopod was stroked there was a slight spreading of the base, with formation of lobes and protuberances and sometimes the appearance of small vacuoles. This response was much more easily elicited by mechanical stimulation of the proximal half of an axopod; usually mechanical stimulation of the distal half had little or no effect.

As a result of a considerable number of preliminary experiments, it was found that individual specimens of *Actinophrys* varied greatly in their response to a fine squirt of water from a micropipette, and also that the use of serum albumin in such experiments led to complications which will be discussed later. Accordingly, methods were standardized as far as possible and the later results obtained with egg albumin will be described first.

A comparison was made of the stimulating action of a micropipette filled with tap water and (immediately afterwards) of the same pipette filled with a solution of crystallized egg albumin ($\frac{1}{2}\%$, 1%, or $2\frac{1}{2}\%$). In any one experiment the same *Actinophrys* was used throughout, and the stimulus took the same form. Either the pipette was placed and left in contact with the body surface proper (without any squirting) or a squirt was given from a distance of $\frac{1}{2}$ or $\frac{3}{4}$ of the radius of the body (without axopods), and the pipette was left in position. If there was no

response to the pipette filled with tap water the stimulus was repeated within a few minutes.

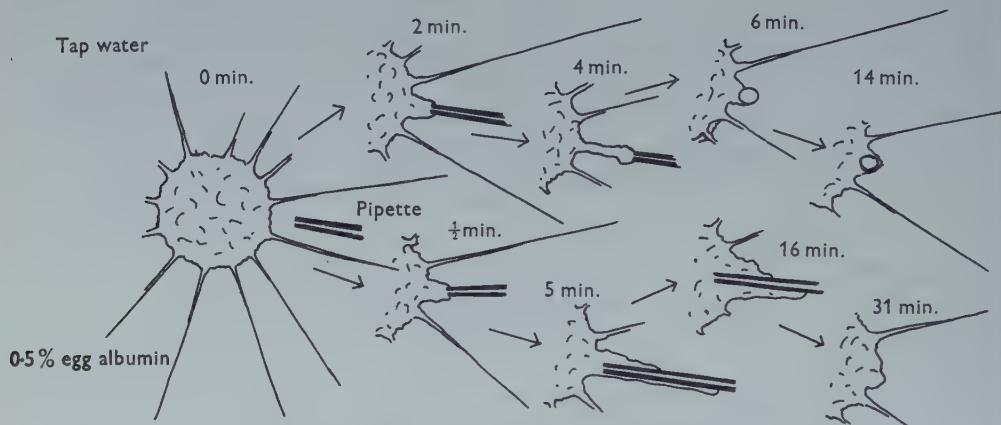
The results of these experiments are summarized in Table 1, and an example is illustrated in Text-fig. 2. With tap water in the pipette, contact with the body surface sometimes provoked the formation of a small cup around the pipette tip, but this was soon withdrawn; the response to squirting was no greater, and usually less. Egg albumin, whether administered by contact or by squirting, provoked a greater response; usually a cup held the pipette and spread a considerable distance up the outside of it, as illustrated in Text-fig. 2. The pipette was then drawn deep into the body. Finally it was extruded, usually $\frac{1}{2}$ –1 hr. after the initial stimulus.

Table 1. Pokes and squirts with pipette loaded with (a) tap water, then
(b) crystallized egg albumin dissolved in tap water

Experiment no.	Tap water	Egg albumin in tap water
061159	2 squirts: (1) no reaction; (2) a small protuberance	$\frac{1}{2}\%$, 1 squirt: local spread of axopod bases
111159a	2 squirts: (1) expansion of axopod base, small funnel captured pipette, released in 5 min; (2) small funnel again captured pipette, released in 4 min.	$\frac{1}{2}\%$, 1 squirt: extensive membranous outgrowth spread far up outside of pipette; pipette then drawn into body; released in 30 min.
111159b	2 squirts: no effects	$\frac{1}{2}\%$, 1 squirt: immediate local spreading of axopod bases; membrane grew out to pipette and held extensively; pipette drawn into body; released in 44 min.
181159	2 squirts: no reaction; 1 touch; adhesion, released in 9 min.	$\frac{1}{2}\%$, 1 squirt: cup immediately held pipette and extended over it. Pipette drawn into body; released in 32 min.
251159	2 touches: adhesion for 2 and 3 min.	$\frac{1}{2}\%$, 1 touch: pipette enveloped immediately, then drawn into body; released in 35 min.
291159	2 squirts: no reaction; 2 touches; no reaction	$\frac{1}{2}\%$, 1 squirt: local thickening; 1 touch, slight cup surrounded pipette; released in 14 min.
300959a	1 touch: small cup surrounded pipette, released in 4 min.	$2\frac{1}{2}\%$, 1 touch: extensive outgrowth over pipette; pipette drawn into body; released in 76 min.
301159b	1 touch: adhesion but no cup, released in 6 min.	$2\frac{1}{2}\%$, 1 touch: extensive outgrowth over pipette; pipette drawn into body; released in 50 min.

After preliminary work, the effects of squirting tap water and then bovine serum albumins fraction V (0.1% or 0.2% in tap water) were compared in fourteen experiments carried out otherwise as described above. Although the response was usually greater to the serum albumin than to tap water, the difference was not enough to carry conviction in view of the variation. The response to serum albumin ranged from the extension of a few small protuberances to the outgrowth of a large and extensive funnel which spread up the outside of the pipette. In two experiments no lobes were formed, but a skin was lifted up from the body surface in the region

of the squirting and moved outwards along its axopods. This phenomenon is described in the next section. Owing to skin-lifting it was not profitable to use higher concentrations of serum albumin.



Text-fig. 2. Diagrams showing the response of *Actinophrys sol* to a squirt of tap water and then of crystallized egg albumin (0.5% in tap water).

IRRIGATION WITH TEST SOLUTIONS

Methods

A number of *Actinophrys* and a little debris from the bottom of the culture were mounted under a cover-glass supported by filter-paper leads. With careful irrigation specimens having a few axopods lodged in debris were left undisturbed; and with extreme care it was possible to use individuals resting only on the glass. Irrigation was carried out by hand throughout an experiment, first with tap water, then with a solution of the test substance, and occasionally with tap water again. All solutions were filtered, in view of the fact that the γ -globulin was found not to dissolve completely.

Results

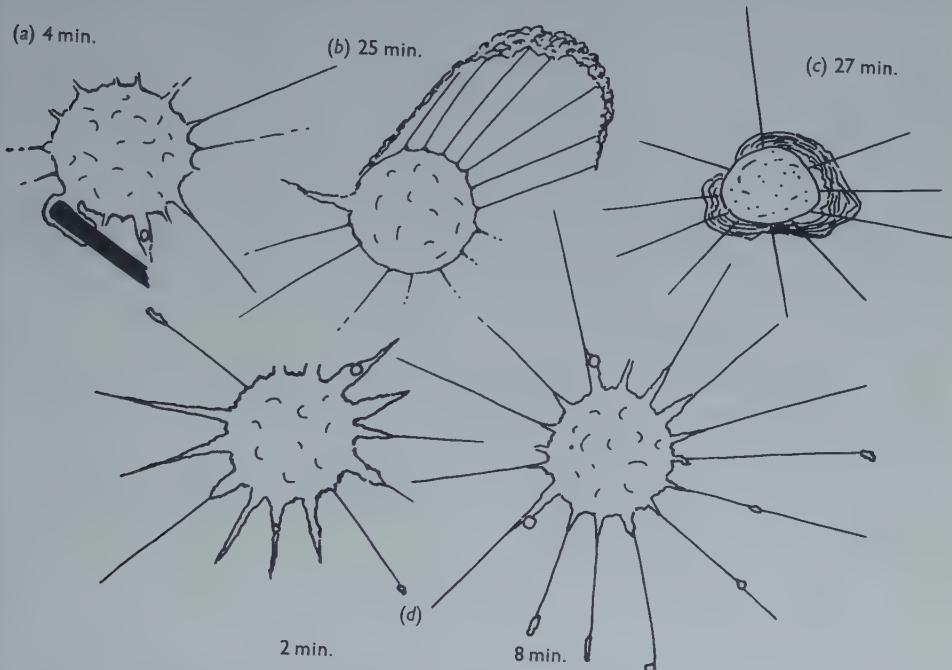
Egg albumin (0.05–2.5% ; 7 experiments with crystallized egg albumin, 11 experiments with flake)

There was an immediate shortening of the axopods, and an expansion and lobulation of their bases (Text-fig. 3), more marked at the higher concentrations. Within a few minutes or less, small vacuoles appeared at the surface of the axopod bases. I did not see where they came from, but I interpret them as pinocytic. After a few minutes the lobulation subsided and no more vacuoles appeared. Often, however, aggregations of material moved outwards along the axopods and were cast off at the tips (Text-fig. 3). In 0.5% egg albumin and upwards, a coherent sheath or skin of similar material often separated from the body surface and moved outwards along the axopods.

No difference was seen in the responses to crystallized and to flake egg albumin.

Serum albumin fraction V. (0·1–5 %, 14 experiments, excluding preliminary work)

There was little immediate change in the appearance of the axopods. 'Skin-lifting' (Pl. I) took place at all concentrations, though not in all individuals in the 0·1 % solution. Skin-lifting was usually first apparent within a few minutes of irrigation with serum albumin solution, although sometimes it was delayed.



Text-fig. 3. Diagram showing the effects of (a) contact with a glass probe at the body surface, and irrigation with (b) 0·05 % γ -globulins, (c) 5 % serum albumin fraction V, and (d) 1 % egg albumin (flake), in four separate experiments.

A sheath or skin appeared to form outside the body surface and began to separate from it, moving outwards along the axopods. Usually the separation started on one side but spread all round the body. The skin was then lifted completely off the body, and travelled outwards along a group of axopods, which were themselves drawn together in the process. At this stage the skin and axopods looked like a parachute. The skin was subsequently shed completely. The first stages of skin-lifting proceeded rather quickly. A skin could move half way out along the axopods within a minute or two, but the later stages occupied $\frac{1}{2}$ –1 hr. or longer. With the higher concentrations (2½ and 5 %) the skin was very much thicker (Text-fig. 3) and did not completely separate from the body during observations ranging up to 5 hr.

After separation of a skin the body surface was much more smoothly rounded, and the axopod bases appeared thinner. The organisms appeared perfectly healthy. When given food immediately after skin-lifting, many captured and ate *Astasia* and *Colpidium*, and some did so even though still partly surrounded by skins.

Normally after mass feeding of a culture of *Actinophrys*, groups of partially fused individuals are found sharing one or more common food vacuoles. On treatment with serum albumin such groups lifted skins, and afterwards the individuals of a group fused more completely, so that the angles between them disappeared and it was no longer possible to distinguish one individual from another.

Crystallized serum albumin has proved just as effective in provoking the lifting of skins, but the experiments concerned with this will be described in a later paper.

γ-Globulins fraction II (0·01 and 0·05%, 11 experiments)

In some cases local thickenings developed on the axopods, as though axopod substance had aggregated, and the tips of the axopods became bent. In some cases also an incomplete thin skin was lifted from the body surface, or small clumps of material appeared at the body surface and travelled quite rapidly outwards along the axopods. In one case a number of funnels grew out on irrigation with a 0·05% solution.

Gelatin (Belgian 'Gold Leaf', 0·1%, 3 experiments)

A few specimens definitely formed skins. No extensive investigation was made.

Peptone (Bacteriological, 0·1-3%, 9 experiments)

Occasionally the axopod bases expanded somewhat, and small vacuoles appeared in them. Out of numerous individuals three developed thin but recognizable skins.

Sucrose (0·01-0·1M, 4 experiments)

No skins were formed, and pinocytic vacuoles were rare.

Toluidine blue ($0\cdot5 \times 10^{-2}\%$ and $0\cdot25 \times 10^{-2}\%$, 10 experiments with toluidine blue only, 4 experiments after skin formation in 0·2% serum albumin fraction V)

Direct treatment with toluidine blue solution led to a dense violet staining of the body surface. In many individuals a violet or violet pink skin was lifted off, leaving the body surface unstained. The body surface was smooth and rounded after the skin had separated.

Skins already induced by serum albumin stained blue on addition of toluidine blue, but later became more violet.

Thionine (1-5% saturate, 9 experiments)

The body surface stained violet, and in some experiments a violet or violet pink skin then lifted off it, leaving it unstained. Sometimes there was no continuous skin, but a diffuse pinkish violet mass or a line of granules. The body surface was rounded and smooth after the separation of this material.

DISCUSSION

The formation of food cups by amoebae in response to the vibrations of a needle or to a capillary containing egg albumin has been described by Schaeffer (1917). It is clear that the outgrowth of a food funnel from *Actinophrys sol* can be caused

by mechanical stimulation, and it appears also that a solution of egg albumin can assist the process. Immersion in a solution of egg albumin causes an expansion and a lobulation of the axopod bases, like that seen locally when dilute egg albumin is squirted at *Actinophrys* from a micropipette. It is not to be expected that combined chemical and mechanical stimulation with a micropipette will produce exactly the same pattern of response as that produced by a captured but still active ciliate. The beating of cilia probably holds the membranes off from the body surface of the prey and results in a much larger food vacuole, and the chemical stimulus provided by the ciliary surface may also be very different in its effects. Nevertheless, it is interesting to compare the mechanism of stimulation in *Actinophrys* with that determining the discharge of nematocysts, for which a combination of the two kinds of stimuli is much more effective (Pantin, 1942). It is possible that in *Actinophrys* also the dual mechanism of stimulation helps to prevent errors in response, although according to Looper (1928) *Actinophrys* eats inanimate particles.

The outgrowth of a food funnel in *Actinophrys* must represent a local excitation, and it is therefore reasonable to suggest that the advancing pseudopod of an amoeba is also in a state of excitation. In view of the efficacy of a biochemical stimulus it is likely that excitation occurs in the surface membrane. The small vacuoles which appear in the surface of the outgrowing lobes and protrusions are no doubt pinocytic. It is therefore likely that chemical stimulation and pinocytosis are closely related, and it is possible that both are brought about by surface adsorption (see Holter, 1959). It would be particularly interesting to know whether any local depolarization is associated with the outgrowth of a funnel of a pseudopod, and if so how this is affected by substances which promote pinocytosis or the formation of funnels.

The nature of the substance which forms the sheath or skin is not yet known, although its metachromatic staining is suggestive. Its origin and nature have not yet been traced. It emerges or separates from the body surface in response to the presence of certain substances, which include the proteins already mentioned; presumably it reacts with proteins. It is probably of the same general nature as that described for *Tetrahymena* by Bresslau (1922) and more especially by Robertson (1939), who showed that a thick sheath is developed in response to an antiserum. However, the skin-lifting of *Actinophrys* is far from specific. Its normal function is not known; it might perhaps serve to stick flagella and cilia, or to inactivate harmful proteins or other substances in the prey, or to hold on to debris during removal of these along the axopods. The rounding up of *Actinophrys* after skin-lifting is particularly interesting, as it suggests that the skin material or layer may normally contribute to cell shape, although it is also possible that the influence is an indirect one.

SUMMARY

1. Various flagellates and small ciliates stick to the axopods of *Actinophrys*. Contact with the base of an axopod or with the body surface leads to the outgrowth of a food funnel, by which the prey is ultimately surrounded.

2. If a fine probe or micropipette touches the body surface or the base of an axopod, a small cup may grow out over it, investing it closely, but the instrument is soon released. A squirt of tap water from a micropipette may also provoke the outgrowth of a small lobe or cup, or local pinocytosis.

3. Contact with, or a squirt from, a micropipette containing a solution of egg albumin provokes a more extensive reaction. The micropipette usually becomes invested extensively. The micropipette is drawn into the body and held there for up to an hour.

4. Immersion in egg albumin solution leads to a temporary spreading and lobulation of the axopod bases, and later a 'skin' may separate from the body surface. Skin formation is more pronounced in serum albumin solution, and may also be induced by γ -globulins and gelatin.

5. On treatment *in vivo* with toluidine blue or thionine a violet layer in the body surface separates as a pinkish violet or violet skin, leaving the body surface unstained.

6. There is evidence that the skin-forming substance is associated directly or indirectly with the maintenance of cell shape.

I am much indebted to Mr E. A. Livingstone for a steady supply of *Actinophrys* and for cultivating the various Protozoa used as food.

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EXPLANATION OF PLATE 7

Actinophrys sol was irrigated with 0·1% serum albumin fraction V. The first photograph was taken 1 min. before, and the others at the time stated after irrigation with serum albumin began. Note the lifting and separation of the 'skin'.



(a) Before treatment



(b) 5 min.



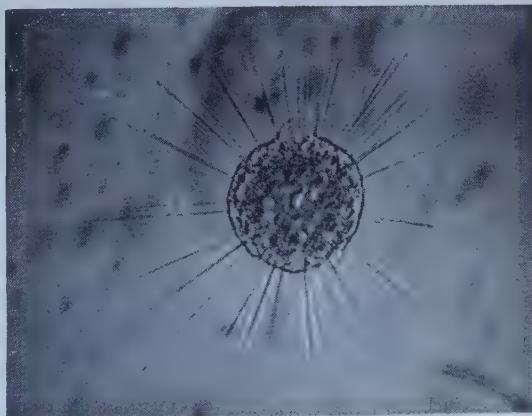
(c) 17 min.



(d) 24 min.



(e) 93 min.



(f) 124 min.



KITCHING—RESPONSES OF THE HELIOZOON *ACTINOPHRYX SOL* TO PREY, TO MECHANICAL STIMULATION, AND TO SOLUTIONS OF PROTEINS AND CERTAIN OTHER SUBSTANCES

(Facing p. 416)

THE RESPONSE OF THE AMPULLAE OF LORENZINI OF ELASMOBRANCHS TO MECHANICAL STIMULATION

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(Received 4 February 1960)

INTRODUCTION

It has generally been assumed since Sand's electrophysiological demonstration of the thermal sensitivity of the ampullae of Lorenzini of elasmobranchs (Sand, 1938) that their function is temperature reception. The simple behavioural experiments of earlier workers (e.g. Parker, 1909; Dotterweich, 1932) had indicated a mechano-receptive function of some sort, but Sand had been unable to detect a response in the nerves to mechanical stimulation, and Hensel found such a massive stimulus necessary (in dogfish) that a mechanoreceptive function seemed improbable (Hensel, 1956).

However the anatomical arrangement of the ampullae and their tubes, especially in rays (Figs. 1 and 2), cannot be understood if their function be thermoreception, since the jelly in the tubes does not appear to have a specially high thermal conductivity (see p. 422). A further investigation of the function of the ampullae was therefore justified, and a preliminary account of this has appeared (Murray, 1957).

MATERIAL AND METHODS

Various *Raja* species were used, mainly *R. clavata*, but occasionally *R. naevus* or *R. montagui*; the results obtained with each species were similar. A few dogfish (*Scylliorhinus canicula*) were also studied. For convenience, the head of the pithed fish was cut off, but when this was not done, the ventral aorta was ligated to prevent the seepage of blood into the dissection cut. The ampullae of the mandibular capsule were used, because of the ease of the dissection and the length of fine nerve available. In early experiments the nerve, capsule, tubes and overlying skin were dissected out, but later the capsule and tubes were left *in situ* and undisturbed except that the skin over the course of the nerve had to be cut away as far as the base of the capsule. This had to be done because it was essential in testing for mechanical sensitivity to cut the nerve from the more sensitive lateral line organs which forms a mixed nerve trunk with the ampullary fibres (Fig. 2A). The cut can only be made reliably distal to the base of the capsule where the lateralis nerve branch is separate from the ampullary, and lies beside the capsule on its way past from the mandibular lateral line canal. The nerve was lifted up over a pair of Ag/AgCl wire electrodes, and strands were separated off successively with scissors and thinned down until a record was obtained with a suitably small number of

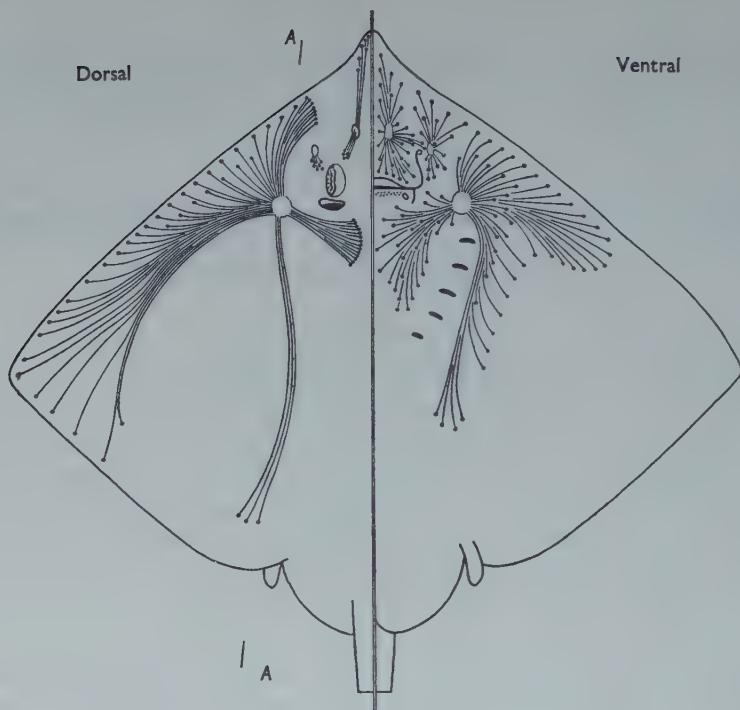


Fig. 1. Diagram showing the extent of the tubes of the ampullae of Lorenzini of an individual *R. clavata*, on the dorsal and ventral surfaces. The tubes are not shown which come from the mandibular capsules just behind the mouth. The other three capsules on each side have tubes opening on both dorsal and ventral surfaces. The section at *A-A* is shown in Fig. 2C.

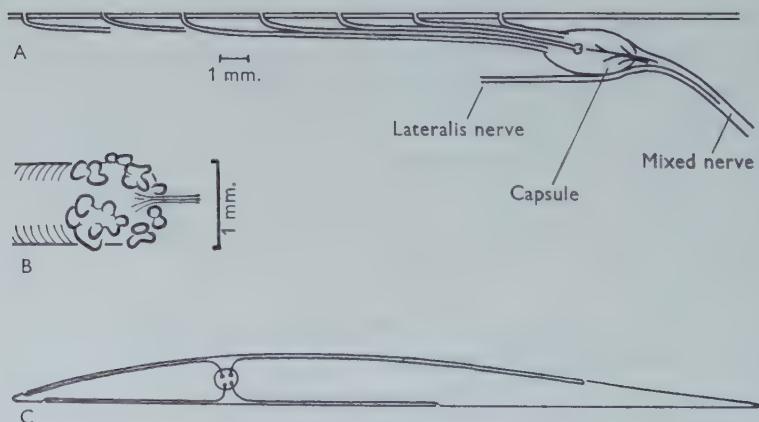


Fig. 2. A. Diagrammatic section of the skin just behind the mouth, showing the mandibular capsule and some of the tubes running from it. The scale refers to a fish of about 50 cm. span. B. An individual ampulla from the hyomandibular capsule of a 50 cm. span *R. clavata*. C. Diagrammatic longitudinal section through the base of the wing (at *A-A* in Fig. 1) showing four of the ampulla tubes and the hyomandibular capsule.

active units. Normally the sensitivity of the preparation would be tested at this stage before the attempt was made finally to isolate a single unit. The process of separation of strands could be repeated along the length of the nerve so that more units could be investigated.

A roughly quantitative assessment of the sensitivity of the preparation was made by using progressively stiffer probes. Initial tests were made with a nylon filament of $30\ \mu$ diameter, which could exert a force of up to the equivalent of 1 mg. If this was inadequate a human hair was used, which exerted a maximal force of the equivalent of 5–50 mg according to the angle at which it was held. Finally, a pointed rod was used. When sensory adaptation was being studied the probe was held in a manipulator.

Nerve impulses were recorded with a conventional a.c. amplifier, C.R.O. and tape-recorder.

RESULTS

The ampullae of Lorenzini in rays are sensitive to very slight mechanical stimulation; when the end-opening of the tube of an ampulla is lightly touched with the nylon filament the resting discharge in the corresponding nerve fibre is briefly speeded-up (Fig. 4A), or a short burst of impulses occurs if there is no resting discharge (Fig. 3). If now another strand of the nerve is used for recording, a different opening is found to be sensitive. In this way, in some preparations, sensitive responses have been obtained from between one-third and one-half of the 20–30 ampullae of the mandibular capsule; this means that on about half the occasions on which the nerve was successfully dissected down to leave only two or three active units a touch-sensitive opening could be found.



Fig. 3. The impulse discharge in a two-fibre preparation showing the brief burst of impulses in the spontaneously silent unit when the corresponding opening is touched with a nylon filament.

The ampullae can easily be overstimulated and their sensitivity reduced or abolished. Touching a previously 'nylon-sensitive' opening with a hair abolishes the response to nylon, at least for a few minutes and often permanently, although the response to a hair may remain. Even 'hair-sensitivity' may be lost if the hair is pushed right into the opening of the tube—a stimulus which produces an impulse discharge at maximal frequency, adapting after several seconds. Because of the ease with which the sensitivity of the organs may be destroyed it is essential that dissection be kept to a minimum. Unfortunately, although the capsule and the tubes can be left intact in their places, the nerve has to be followed up to the capsule so that the lateralis branch may be cut, and this involves some disturbance of the capsule. It is not therefore surprising that many preparations showed reduced sensitivity.

So far, reference has been made only to excitatory effects. Touching the opening results usually in an increase in the frequency of the resting discharge, followed by an inhibitory after-effect when the stimulus is removed (Fig. 4). But often the initial effect is inhibitory, the discharge being slowed down or even stopped during the touch, with a post-inhibitory rebound (Fig. 5). The sensitivity of the two kinds of response is similar. Sometimes an active unit which is speeded up by a touch on one opening may be slowed by touch on the skin alongside or at an adjacent opening.

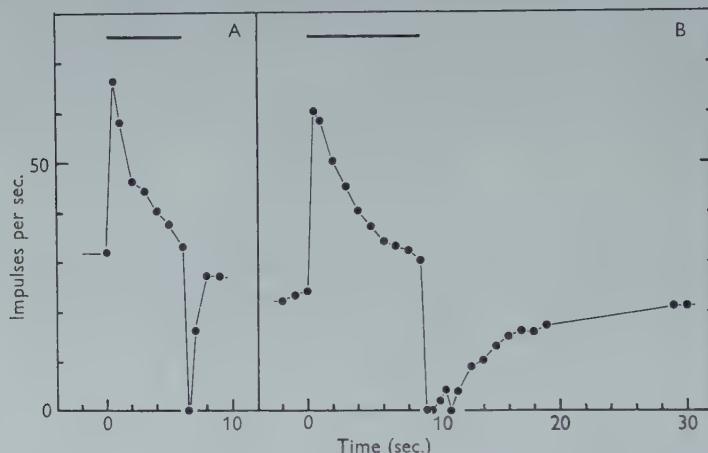


Fig. 4. Discharge frequencies of two preparations in response to touch. The duration of the stimulus is marked by the horizontal line. (Stimulus in A, nylon; in B, rod.)

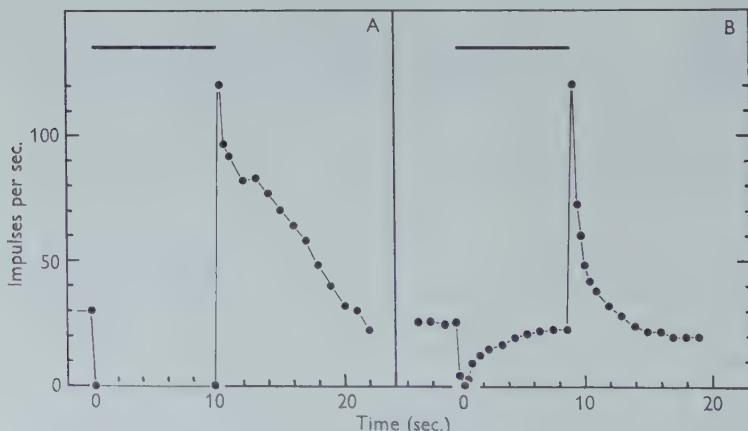


Fig. 5. Discharge frequencies of two preparations in response to touch. The duration of the stimulus is marked by the horizontal line. (Stimulus in A, nylon; in B, rod.)

In a series of 15 consecutive preparations (10 of them isolated, 5 *in situ*) 32 nylon-sensitive single units were identified in which the frequency was increased, and 15 in which it was decreased by touching an opening. There were also 19 units

which were speeded by a hair touch, and 4 which were inhibited. The remaining small multi-fibre or single-unit recordings, in which a response to touching an opening could not be elicited, could almost all be excited and inhibited by touching or distorting the region where the tubes emerged from the capsule.

Typical response curves (Fig. 4, 5) show that the process of 'adaptation' is total and relatively rapid, the discharge frequency returning three-quarters of the way back to its initial level in 3–8 sec. However, it is not possible to state that this is a genuine process of adaptation in the sensory nerve ending as it may result in part from mechanical accommodation in the sense organ reducing the effectiveness of the stimulus. For instance, the jelly from the ampullae behaves elastically to sudden pressures, but flows plastically under maintained pressure. In a few preparations (Fig. 6) in which the reduced sensitivity necessitated stronger stimulation such as firm pressure with a blunt rod over the centre of the extent of the tube, the adaptation time was as long as 20–30 sec., which is similar to that found with electrical stimulation (Murray, 1959).

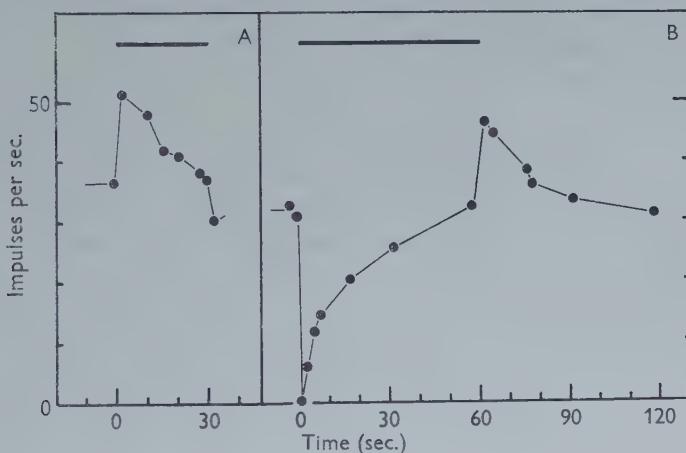


Fig. 6. Discharge frequencies of two preparations in response to maintained firm pressure on the tubes showing the longer adaptation times. The duration of the stimulus is marked by the horizontal line.

Other methods of mechanical stimulation have been observed qualitatively to be effective. If the whole preparation is immersed in sea water, a pipette-jet directed against the opening, strong enough to cause a small visible indentation in the skin, results in a detectable change in impulse frequency. Movement of the skin surrounding the capsule and tubes is also effective: for example, pushing the lower jaw 0.5 mm. upwards (morphologically) or pulling the skin at a point 20 mm. posterior to the organs so that the region of the openings moves 0.2 mm., are both stimuli strong enough to cause complete inhibition of the resting discharge (the opposite movement of course causes increase in frequency). In view of Dotterweich's suggestion that the ampullae are depth receptors, the effect of changing

the depth of the shallow water covering the preparation was studied in a few experiments. No clear results were obtained as although responses did sometimes occur the possibility of movement in the preparation could not be excluded.

Experiments on the mandibular capsules of the dogfish (*Scylliorhinus canicula*) show that they also are mechanically sensitive, but because the openings are hidden beneath the scales, hair or nylon testing is not possible.

However in one preparation, for example, pressure with a light plastic rod near the centre-line of the fish just behind the lower lip was excitatory, and pressure on the skin just beside the front end of the capsule was inhibitory for one particular unit, and the impulse discharge in another was speeded by touch two-thirds of the way to the centre-line along the line of the tubes while pressure just anterior to the fold at the corner of the lip was inhibitory.

In connexion with the possible thermoreceptive function of the ampullae the changes of temperature inside one of the hyomandibular capsules and in muscle at a similar depth in the body of a pithed ray were compared following a sudden change in the temperature of the surrounding water. Thermocouple junctions embedded in hypodermic needles were used. No evidence was obtained of a more rapid change of temperature in the capsule than in the muscle.

DISCUSSION

From their nature, electrophysiological experiments cannot demonstrate the functional importance of a sense organ in the life of an animal. Only if there is a single, clearly most sensitive, modality of stimulation can the function be inferred. This is certainly not so in the case of the ampullae of Lorenzini, where responses sensitive enough to be the basis of a sensory function have been recorded for thermal (Sand, 1938) as well as mechanical stimuli. Moreover, experiments in progress indicate that the ampullae are sensitive to voltage gradients in the water as small as $2 \mu\text{V}/\text{cm}$. (Murray, unpublished; see also the hypothesis of Lissmann, 1958). The real function of the ampullae therefore remains uncertain. The movements of the body of the fish which occur in swimming or feeding will be effective stimuli and so also will contact with the substrate. But the fish has other receptors which between them could cover these functions adequately, such as the muscle stretch receptors (Fessard & Sand, 1937), the dermal pressure receptors (Lowenstein, 1957) and the cutaneous free nerve endings. Like the lateral line organs, which are also sensitive to distortion of the skin resulting from the fish's own movements, but which have in addition a well-circumscribed special function (see Dijkgraaf, 1952), the ampullae must presumably have some distinctive function, and it is reasonable in view of their anatomy and central location to suppose that accurately timed comparisons between what is happening at the ends of the tubes will be important. Evaluation of the changing hydrodynamic pressure distribution over the surface of the aerofoil-like body when swimming or gliding could be such a function.

The following calculation indicates the magnitude of the pressures involved (see Goldstein, 1938). The greatest positive increase in pressure which can occur

at the front of an aerofoil (at the stagnation point a little way back on the under surface) is $\rho V^2/2g$ where ρ = density of the medium, g = the acceleration due to gravity and V = velocity. In a conventional aerofoil, and especially in one as thin as a ray's wing, the decrease in pressure just behind the leading edge may at suitable angles of incidence be as much as four or five times greater than $\rho V^2/2g$. If the angle of incidence changes suddenly, it is reasonable to suppose that changes of pressure could occur of about twice $\rho V^2/2g$, either increase or decrease. If a speed of 100 cm./sec. is assumed, the pressure changes would be approximately 10 g./cm.². Now the area of the opening of the mandibular ampulla tubes is 10^{-4} cm.² and therefore the changes in force applied to one ampulla would be 10^{-3} g. This is the value found to be effective experimentally.

The transducing mechanism, whereby the mechanical stimulus is converted into a change of nerve impulse frequency, also remains obscure. It is clear that stimuli which raise the pressure of the jelly inside the ampulla relative to the general pressure in the capsule are normally excitatory, and that inhibition is caused by stimuli which lower the relative ampulla pressure, as for example skin distortion which stretches the tube, or even touch on an adjacent hole which increases the local capsule pressure. But it may also be that there are specific 'inhibitory' nerve endings, in which an increase of ampulla pressure results in a decrease of frequency. A double innervation is possible anatomically, since about six fibres run from each ampulla.

Sand's inability to detect a response to mechanical stimulation can be attributed to the reduction in sensitivity which occurs when the preparation is disturbed and in particular when it is dissected away from the body. The low sensitivity of Hensel's isolated dogfish preparation (he had to press hard on the capsule wall to get a response, which incidentally was inhibitory) was probably also due to this kind of change. In fact, threshold sensitivity tests are of little value when made on isolated, and so possibly damaged, preparations. But Sand also recorded *in situ* without much dissection, but from the whole nerve, and in this case the mechanical stimulus would have caused as many fibres to be inhibited as were excited and so the response might have been overlooked. Where a stimulus may cause either speeding or slowing of the discharge, it is essential to reduce the complexity of the recording until the response of individual units can be recognized.

SUMMARY

1. The ampullae of Lorenzini are sensitive to weak tactile stimulation applied to the ends of their jelly-filled tubes.
2. Either an increase or a decrease in their resting discharge frequency may be caused, each with an opposite after-effect.
3. 'Adaptation' is total, being three-quarters completed in 3–8 sec. This 'adaptation' probably includes accommodative changes of the tissues.
4. The function of the ampullae is discussed, but no definite conclusion can yet be reached.

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THE DEVELOPMENT OF SALINITY TOLERANCE
IN THE SALMON, *SALMO SALAR* (L.) AND
SOME RELATED SPECIES

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INTRODUCTION

The freshwater life of a young salmon begins with the hatching of the egg, and after a variable number of years culminates in the seaward migration of the smolt. During this time the young salmon changes in size and behaviour, from fry to parr and from parr to smolt. This latter transformation is identified with very marked changes in appearance and behaviour. This change has been studied intensively in both the genus *Salmo* and the genus *Oncorhynchus*, especially with regard to the extrinsic factors, to behaviour, and to the hormonal balance (Hoar, 1953; Fontaine, 1954). While there are many outward manifestations of the parr-smolt change known, and a good deal about the behavioural changes and probably related hormonal changes associated with the transformation, there are many unexplained phenomena in the physiology of such fish.

One of the most spectacular changes taking place during the juvenile life of the salmon is the development of mechanisms for osmoregulation in both fresh water and sea water. The ability to tolerate environmental changes is a faculty which may have developed over the whole period of juvenile life, or it may have appeared as a sudden change prior to the parr-smolt transformation and the seaward migration. That is, some ability to tolerate environmental changes may always be present, increasing with age; or it may be latent until a 'triggering-off' by some unknown stimulus sends the smolt downstream to the sea. Thus it seemed that a study of the 'salinity tolerance' of young salmon would provide information relevant to a study of the seaward migration. Three principal questions arose:

- (1) What is the degree of tolerance to different salinities at each stage in the life history and how does it compare with that of other fish?
- (2) If there is a difference in the tolerance of parr and smolts, does this come about suddenly with the other manifestations of the parr-smolt change, or does it develop continuously during the juvenile life of the salmon?
- (3) Is there any pre-migratory adaptation, in 'pre-smolts' or smolts, for marine life?

The following study was undertaken in an attempt to provide information on these questions.

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METHODS

Three species of the genus *Salmo* have been used: *salar* (L.), *trutta* (L.) and *gairdnerii* (Richardson), all hatchery-reared fish of different ages and sizes. Although coming originally from both hard and soft waters, these experimental fish had been maintained for some weeks in a hard unchlorinated water in standard laboratory conditions. Smolts of *Salmo salar* were an exception to this; these fish were naturally reared, caught on or just prior to their downstream migration and were investigated in the field by the same methods as were used in the laboratory.

For each experiment starved fish were used in batches of ten, or duplicate batches of five for larger fish, in 40 l. static aerated tanks at constant temperature (10–12° C.) and oxygen (8–9 p.p.m.). The water was changed frequently to avoid accumulation of waste products and to ensure that the oxygen level was constant.

Salinities used were appropriate to full strength Bay of Biscay sea water (designated 100% sea water = 33·9‰ sal.; Oliver, 1957) and dilutions of this sea water were made with unchlorinated hard water (75, 50 and 25% sea water). The control fish were kept in 40 l. tanks of fresh water. Experiments with the newly hatched alevins were made with batches of 20 in litre beakers suspended in a water bath to maintain the standard conditions of temperature.

Tolerance to salinity changes was measured in two ways. First, by observing the experimental fish and recording the times of their deaths, so that survival times could be considered in relation to salinity. In this case the figures of survival of the fish are expressed in terms of the median survival time, the median being a useful parameter especially where all the fish did not die, or where some survived for much longer periods than the others. Secondly, much more precise information was obtained by measuring the freezing-point depression of the blood of the experimental fish. The blood samples were taken by cardiac puncture, without anaesthetic. Very small quantities of blood were taken from the fish (less than 10^{-3} ml.) and samples for freezing-point (10^{-6} ml.) used with Ramsay's cryoscopic apparatus (Ramsay, 1949; Ramsay & Brown, 1955). The freezing-point depression (Δ° C.) of the blood of individual fish was recorded 1, 2, 4, 8 and 24 hr. after the beginning of each experiment, and then on subsequent days. These measurements indicated whether the fish was able to regulate in the experimental medium, or only able to tolerate the increased salinity, and they also gave some measure of the time required by each group of fish to accommodate to the conditions of the experiment.

RESULTS

Considering first the survival of young *Salmo salar* in the experimental salinities (Table 1 and Fig. 1), it can be seen that in all the batches of fish survival is complete in control conditions (static fresh water) and that it is reduced as the salinity of the experimental medium is increased. Taking fry (3 months old) as an example: these fish were all able to survive a salinity of 25% sea water for the duration of the experiment, but when the salinity of the medium was increased to 50% sea water

(and thus hypertonic) the median survival time was only 7·0 hr. The median survival time in 75% sea water was reduced further to 4·0 hr., and in 100% sea water was only 2·0 hr. The same decline in survival with increasing salinity can be seen in the figures for parr and smolts. An exception to the general trend is shown by the figures for newly hatched alevins. These fish showed very similar survivals in 25 and 50% sea water (with a median survival time of 45 hr. in each case), with

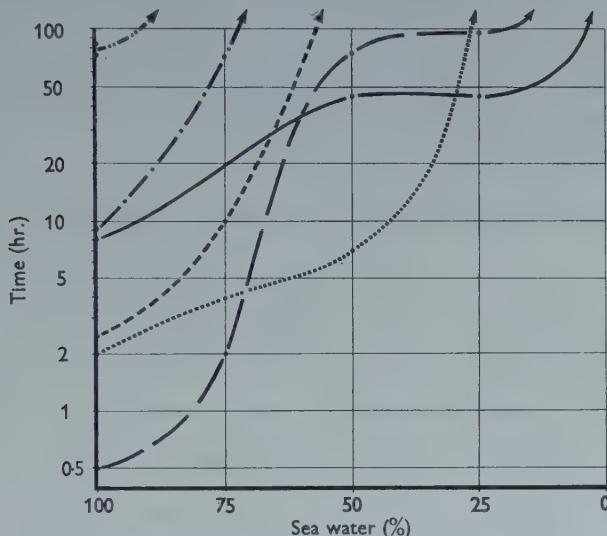


Fig. 1. *Salmo salar* relative survival in sea-water dilutions at different ages and sizes. Alevins, 1 week old, —; alevins, 6 weeks old, - - -; fry, 3 months old,; parr (1), 7 months old, - - - -; parr (2), 7 months old, - - · - -; smolts, 2+ years old, - - - - -.

Table 1. Relative survival of *Salmo salar* at different ages

Stage in life-history	Size range (cm.)	Age	Median survival time (hr.)				
			100 % sea water	75 % sea water	50 % sea water	25 % sea water	Control
Alevins	—	1 week post-hatch	8·0	19·0	45	45	∞
Alevins	—	6 weeks post-hatch	0·5	2·0	76	96	∞
Fry	1·5-2	3 months	2·0	4·0	7·0	∞	∞
Parr	3-4	9 months	2·5	10	∞	∞	∞
Parr	7-10	9 months	9·0	72	∞	∞	∞
Smolts ex R. Lledr ex R. Coquet (migrating)	12-15	2+ years	72	∞	∞	∞	∞
	12-15	2+ years	84	∞	∞	∞	∞

declining survival times in the higher salinities. The greater survival time of the 1-week post-hatch alevins is notable compared with that of fry in all the experimental salinities, and a similar increase in survival in 25 and 50% sea water in the 6-week post-hatch alevins. Whether this increased survival of the alevins represents a tolerance of internal salinity changes or a resistance to external ones will be discussed further below.

In the stages of fish chosen, from fry to migrating smolts, there is a continuous increase in survival time in the experimental media, as the fish gets older. There is some indication that this increase in survival is related to an increase in the size of the fish. Two groups of *Salmo salar* parr, one containing fish of 3–4 cm. length, the other fish of 7–10 cm. length, show clear differences of survival time, the larger fish having the higher values. These two groups of fish were of the same age and had been reared in a single tank in a hatchery, the size groups being separated only a week or two before the experiment. Migrating smolts (from two natural freshwater environments) showed a greatly increased tolerance to sea-water dilutions. Only in full strength sea water was there any mortality, and while a figure for median survival (and thus a 50% kill) could be obtained in some experiments, in others more than 50% of the fish survived the direct transfer.

Table 2. *Relative survival of Salmo trutta and S. gairdnerii at different ages*

Species	Size range (cm.)	Age	Median survival time (hr.)				Control
			100% sea water	75% sea water	50% sea water	25% sea water	
<i>S. trutta</i>	8–10	9 months	7·5	76·0	∞	∞	∞
<i>S. trutta</i>	12–15	2 years	11·5	76·0	∞	∞	∞
<i>S. trutta</i>	19–20	> 3 years	36·0	∞	∞	∞	∞
<i>S. gairdnerii</i>	8–10	9 months	7·5	18·7	∞	∞	∞
<i>S. gairdnerii</i>	15–20	> 2 years	120·0	∞	∞	∞	∞

Similar experiments were made with different groups of the related species, *S. trutta* and *S. gairdnerii* (Table 2). In these species, the same conclusion could be reached, that salinity tolerance increases with increasing size of the fish. Thus *S. trutta* of 8–10 cm. size survived less well than those of 19–20 cm. size; and similarly for the two groups of *S. gairdnerii*. In comparing the results for all three species, it will be seen that within any one age group (with slightly differing size range) there is some indication of a species difference. Thus, in parr of the three species (all about 9 months old) the survival order is

$$S. \text{ } salar > S. \text{ } gairdnerii > S. \text{ } trutta$$

and in 'smolts' of the 2-year age group,

$$S. \text{ } gairdnerii > S. \text{ } salar > S. \text{ } trutta.$$

The relative change in the position of *S. gairdnerii* in this series is interesting; it should be noted that the fish of this species used in the experiments are larger in size than those of the other two (although reared in very similar hatchery conditions), and that this size difference is more marked in the 2-year age group than it is in the earlier one. This observation thus confirms the conclusion drawn from the comparison of survival of the two groups of salmon parr.

A diagram illustrating the relative survival of all these groups of fish, together with comparative results for two stenohaline freshwater fish (Herbert & Mann, 1958), is shown in Fig. 2. Generally speaking, the survivals can be plotted as a series of parallel curves, with *S. gairdnerii* (15–20 cm.) on the extreme left with the

greatest survival time, and freshwater roach and perch on the right with the lowest survival. While *S. salar* smolts show a high relative survival, there is very little to distinguish *S. trutta* (8–10 cm.) and *S. salar* (7–10 cm.) at the parr stage, while *S. trutta* of larger size and 2-year age group, seem to be more stenohaline in behaviour. The curve for the alevins shows quite a different pattern, with a sharp break in the curve, indicating a relatively good survival in the lower salinities, but very limited survival in 75 and 100% sea water.

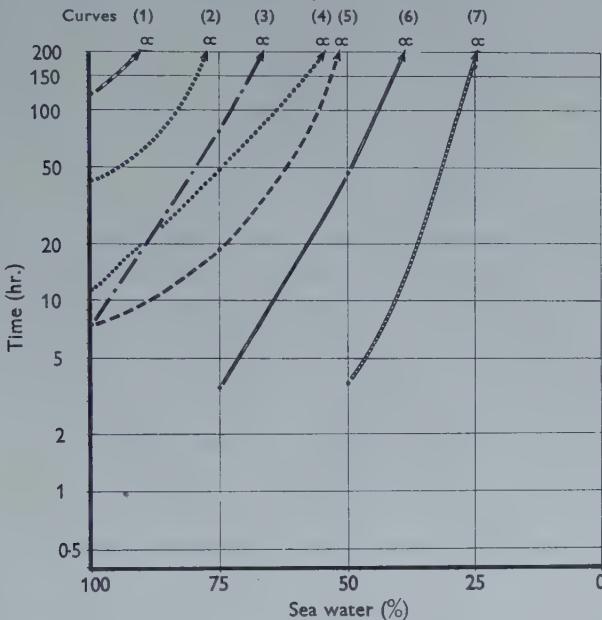


Fig. 2. Relative survival in sea-water dilutions of some teleost fishes. Curves (1) *Salmo gairdnerii* (15–20 cm.); (2) *S. trutta* (19–20 cm.); (3) *S. trutta* (12–15 cm.); (4) *S. trutta* (8–10 cm.); (5) *S. gairdnerii* (8–10 cm.); (6) *Perca* (8 cm.); (7) *Rutilus* (9 cm.).

In addition to observations on survival, freezing-point measurements of the blood of individual fish were made and these give some indication of the degree of osmotic control of which the fish is capable. A summary of these results is given in Table 3, where is tabulated the time taken for fish to return to the 'normal' range of blood concentration.

Measurements of the freezing-point depression of the blood of the three species of salmonids in freshwater conditions show a limited range of concentration. Figures for 1- to 2-year-old fish are shown below:

Salmo salar $\Delta 0.60 \pm 0.04^\circ\text{C}$. ($n = 24$). Range 0.50 – 0.65°C .

S. trutta $\Delta 0.57 \pm 0.02^\circ\text{C}$. ($n = 33$). Range 0.53 – 0.61°C .

S. gairdnerii $\Delta 0.55 \pm 0.03^\circ\text{C}$. ($n = 30$). Range 0.49 – 0.62°C .

When these fish are acclimatized to sea water the blood concentration (measured by freezing-point depression) rises by about 10% of the freshwater values.

Table 3. Time required for blood regulation

Species	Size range (cm.)	Age	Time (hr.) to return to normal blood concentration in		
			100 % sea water	75 % sea water	50 % sea water
<i>S. salar</i>	3-4	9 months	*	*	200
<i>S. salar</i>	7-8	9 months	*	900	200
<i>S. salar</i>	12-15	> 2 years	24	24	4
<i>S. trutta</i>	8-10	9 months	*	300	300
<i>S. trutta</i>	12-15	> 2 years	*	150	100
<i>S. trutta</i>	19-20	> 3 years	*	150	80
<i>S. gairdnerii</i>	8-10	9 months	*	400	150
<i>S. gairdnerii</i>	15-20	> 2 years	150	250	25

* All fish in experiment died.

After introducing the fish into a salinity higher than the fresh water in which it had been living the initial response was a rise in the blood concentration. In the lower experimental salinities the fish usually controlled this rise quite quickly and brought the blood concentration back to the normal level. In the higher external salinities this regulation took longer, and in the case of the smaller size-groups did not occur at all. Thus the time taken to regain control (as expressed in Table 3) gives some measure of the osmoregulatory ability of the fish. These estimates of osmoregulatory ability in general confirm the conclusions drawn from a study of the median survival times, but there are some interesting differences. For instance, the two most 'successful' groups, *S. salar* and *S. gairdnerii* (about 2 years old), both regain control of the blood concentration when in a medium of full strength sea water, but although the median survival time for *S. gairdnerii* is greater than that shown for *S. salar*, the young salmon require the shorter time to control the level of blood concentration in these conditions. Thus the order of osmoregulatory ability for the three species would be

$$S. \text{ } salar > S. \text{ } gairdnerii > S. \text{ } trutta.$$

This difference could indicate that the increased survival time in the rainbow trout depends upon tolerance or resistance to the external conditions, rather than on the ability or activity of the osmoregulatory mechanisms. The order of osmoregulatory ability in the smaller 9-month-old fish of the three species is not so clear.

The internal changes which take place after the fish are put into sea water, or its dilutions, can be seen expressed graphically in Fig. 3. Here the blood concentration changes in individual salmon of different age groups, after immersion in 100% sea water, are expressed in terms of the freezing-point depression of the blood. It can be seen that the blood concentration rose in all groups, within 30 min. of the transfer. In 6-week alevins or fry this rise continued unchecked, until within 2 hr. the blood concentration rose to more than $\Delta 1.00^{\circ} \text{C}$. After such a rise the fish always died within a variable period. Parr (7 months old) and the younger group of alevins showed a somewhat lower rise in blood concentration but reached the lethal limit of blood concentration in about 5 hr. Only smolts could survive

the direct transfer to sea water, and after an initial rise in 2 hr. to a level greater than the normal, the level of concentration was reduced and relatively constant within 4 hr.

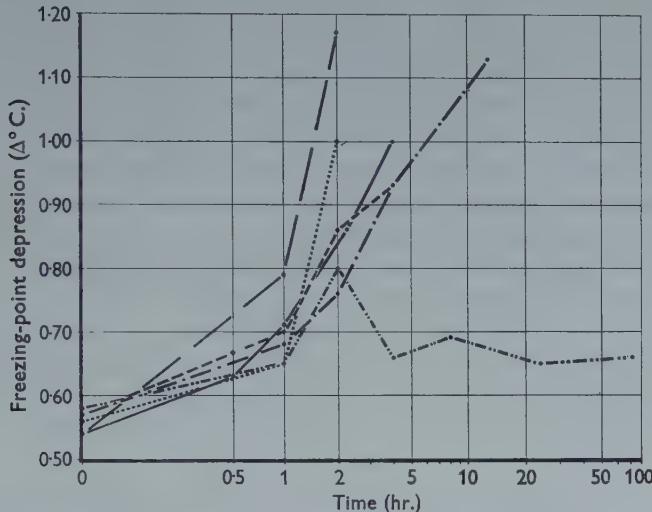


Fig. 3. *Salmo salar*: changes in blood concentration after immersion in full strength sea water.
Key as in Fig. 1. Each point refers to an individual fish.

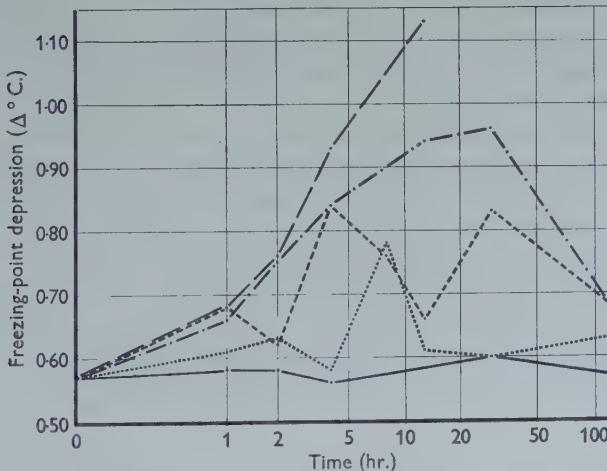


Fig. 4. *Salmo salar* parr: changes in blood concentration after immersion in sea water dilutions.
Control, —; 25 % sea water, ····; 50 % sea water, - - -; 75 % sea water, - · - -; 100 % sea water, ——. Each point refers to an individual fish.

A comparison of the osmoregulatory ability of salmon parr and smolts may be made by comparing the results expressed in Figs. 4 and 5, where the blood concentration changes following immersion in the various media are shown. Parr in 100% sea water lost control of the blood concentration immediately, and in 1 hr.

the concentration of the blood rose to its usual sea-water level ($\Delta 0.67^\circ \text{C}$.); but in 2 hr. it had risen still further and so on, until in 10 hr. it was greater than 1.10°C . In 75% sea water there is a similar but not quite so steep rise in blood concentration, and the fish was able, after about 24 hr. and a rise to $\Delta 0.95^\circ \text{C}$., to bring sufficient regulatory processes into action to reduce the blood concentration to the sea-water level. In 50% sea water the rise in blood concentration is not nearly so great, although the variability of the results does indicate that the fish are in considerable stress. In 25% sea water there is again some variability in blood concentration, even though this medium is hypo-osmotic to the blood. Determination of freezing-point of the blood of control fish taken at the same time show that the individual variations do not go beyond the usual range for these fish living in fresh water. In Fig. 5 similar results for salmon smolts are shown. These fish were 1 year

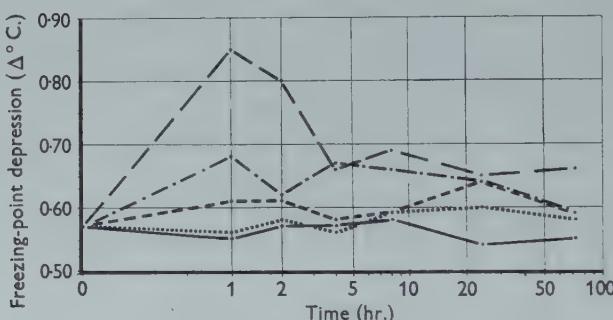


Fig. 5. *Salmo salar* smolts: changes in blood concentration after immersion in sea water dilutions.
Key as in Fig. 4. Each point refers to an individual fish.

older and about 4–5 cm. larger than those of the parr group just discussed. It is immediately clear from these figures (drawn to the same scale) that the regulation shown by the smolts is very much better than that shown by the parr. Not only did these fish survive the transfer to 100% sea water, but the rise in blood concentration following immersion in sea water was not so high, and was relatively quickly reduced (after about 4 hr.) to the normal level for marine fish. The fish in 75% sea water did not even show the initial rise beyond the normal level but only a rise to about the normal level for marine fish. The variation of the blood concentration in these fish in 50 and 25% sea water is scarcely evident, and much less than that shown by the parr.

DISCUSSION

The results of the experiments described above allow some conclusions to be drawn about the ability of these salmonid fish to survive a sudden transfer to sea water. Both survival and the ability to osmoregulate in different salinities depends upon the size and age of the fish, as well as on the species. It may be concluded from the evidence put forward here that survival and osmoregulation are better in larger fish, or in older fish, although if these two factors can be distinguished size appears to be the more important. Within the three salmonid species used for these experi-

ments the salmon seems to survive salinity changes better than the other two species, and the rainbow trout better than the brown trout.

The behaviour of the two age groups of salmon alevins described in this paper is very different from that for the older fry. It is possible that osmoregulation is quite different in these larvae. The epithelium of the alevin is singularly well supplied with mucous cells (Parry, 1958). While the mucus produced cannot by itself maintain an osmotic barrier, is it possible that the epithelium as a whole can maintain some degree of impermeability? The general pattern of the results obtained with alevins could indicate such an impermeability, which breaks down after a time interval almost irrespective of the external salinity.

The increase in survival in the groups of fish, discussed above, could be the result of several factors. First, the effect of an increase in size could indicate that the surface:volume ratio is important, a larger fish being subjected to a lower osmotic stress. Secondly, as the fish gets older it is possible that the skin becomes thicker and less permeable, although osmotic exchanges through the thin and vastly convoluted respiratory epithelium of the gills must completely overshadow any exchanges via the rest of the surface. Thirdly, as the fish grows older there is also the possibility that some salt-regulatory mechanism becomes more efficient. If an increase in the number of acidophil ('Keys-Willmer') cells in the gills could be associated with an increased efficiency in osmoregulation, this would be valuable evidence that this factor was important. However, although there is evidence that salt-loading induces the development of such cells in goldfish and guppies (Liu, 1942; Vickers, 1958), and that injections of thyroid will also induce their development, along with the silverying characteristic of the smolt change (Hoar, Black & Black, 1951), there is no clear and unequivocal evidence that these cells do, in fact, salt-regulate (Parry, Holliday & Blaxter, 1959). Acidophil cells are present from a very early stage (in 4-week alevins); they are more numerous in larger and older fish, but what is needed is to know if they increase out of proportion to the osmotic work required in fresh water.

The growth of a tolerance to salinity is of great importance in the ecology of salmonids, and especially in the case of *S. salar*. It would appear justified from the evidence of these experiments to conclude that those fish which grow quickly are those which develop more quickly a tolerance to sea water. In a population of young salmon the fastest growing parr are thought to be the first to become smolts and to be first to the sea (Pyefinch, 1955). These fish will best be able to withstand such a salinity transfer. If rapid freshwater growth is related to rapid marine development, these fish will complete the life cycle in a shorter time and thus provide a quicker return to a river for spawning. The establishment of this hypothesis by further experimentation could have far-reaching ecological implications.

SUMMARY

1. A study has been made of the survival and osmotic regulation of young salmonid fishes following transfer from fresh water to various dilutions of sea water.

2. Survival is in the order:

$$Salmo \text{ } scalar > S. \text{ } gairdnerii > S. \text{ } trutta$$

and is generally better the larger the fish.

3. The survival pattern of alevins differs from that of the older stages.

4. Hypo-osmotic regulation is first seen in parr and becomes fully effective in smolts.

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